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Liquid formulation of interferon-beta
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Abstract (Basic): WO 9915193 A1

NOVELTY - Stable interferon-beta (I) solution of neutral or slightly acid pH (pH 5-8) is new.

DETAILED DESCRIPTION - Liquid formulations of human (I), that retain at least 80% of in vitro biological activity after storage for 3 months at 25 degrees Centigrade, contain up to 25 MU (units)/ml of (I), are buffered to pH 5-8, preferably over 5.5, and are free of human serum albumin (HSA); are buffered to pH 6-7.2 and are free of HSA, or are buffered to pH 5-8, preferably over 5.5, and contain at least one amino acid.

ACTIVITY - Antiviral; antiproliferative; immunomodulatory.

MECHANISM OF ACTION - None given.

USE - Interferons are known as antiviral, antiproliferative and immunomodulatory agents.

ADVANTAGE - The formulations have high stability (of biological activity and of molecular and physical integrity); eliminate the expense of freeze-drying and reconstitution, and do not require potentially hazardous additives (serum albumin or detergents). They may even be stored for a month at 37 degrees Centigrade and still retain at least 70% of biological activity. A formulation, of pH 7, contained 50 mM sodium phosphate, 50 mg/ml glycerol, 2 mM methionine and 12.5 MU/ml (I). After 6 months storage at 25 degrees Centigrade, recovery of biological activity (assessed conventionally by inhibition of the cytopathic effect of a virus) was 13.4 MU/ml, i.e. 107.2% of the initial value and 172% of a control that had been stored for 6 months at -20 degrees Centigrade.

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# Liquid Interferon-β Formulations

Description

The present invention relates to liquid formulations of human interferon-β. These formulations are characterized in that they have a pH value between 5 and 8 in the weakly acidic to neutral range, and they have a high stability of the interferon-β in solution while retaining its molecular integrity.

Naturally occurring interferons are species-specific proteins, in some cases glycoproteins, which are synthesized by various cells in the body after induction with viruses, double-stranded RNA, other polynucleotides and antigens. Interferons have numerous biological activities such as antiviral, antiproliferative and immunomodulatory properties. At least three different types of human interferons have been identified; these interferons, which are produced by leukocytes, lymphocytes, fibroblasts and cells of the immune system, are referred to as α-, β-, and γε-interferons. Individual types of interferons may also be subdivided into various subtypes.

Native human interferon-β can be produced industrially by superinduction of human fibroblast cell cultures with poly-IC and subsequent isolation and purification of interferon β by chromatographic and electrophoretic techniques. Proteins or polypeptides having properties comparable to those of natural interferon-β can also be produced by recombinant DNA technologies (European Patent Application EP-A-0 028 033; European Patent Application EP-A-0 041 313; European Patent Application EP-A-0 070 906; European Patent Application EP-A-0 287 075; Chernajovsky et al. (1984), *DNA*, 3, 297-308; McCormick et al. (1984), *Mol. Cell. Biol.*, 4, 166-172). Recombinant human interferon-β can be produced in both eukaryotic cells (e.g., CHO cells) and prokaryotic cells (e.g., *E. coli*). The corresponding interferons are known as interferon-β-1a and interferon-β-1b. In contrast with interferon-β-1b, interferon-β-1a is glycosylated (Goodkin (1994), *Lancet*, 344, 1057-1060).

Therapeutic use of interferon-β presupposes that it is converted to a pharmaceutical preparation which permits storage of the protein for a long period of time while maintaining its molecular integrity. Interferon-β is unstable and is subject to various degradation reactions, including in particular, cleavage of peptide bonds, deamidation, oxidation of methionine to methionine sulfide, disulfide exchange and changes in the sugar side chains up to and including deglycosylation.

On the basis of the therapeutic benefit of interferons, a number of formulations have been developed in past years, but they all had certain disadvantages. U.S. Patent No. 4,647,454 (Inter Yeda Ltd.) describes a formulation of fibroblast interferon-β which can be stabilized by adding polyvinyl pyrrolidone (PVP) in the strongly acidic range (pH 3.5). Other preferred additives include mannitol, human serum albumin and acetate buffer. The formulation is freeze-dried and stored at 4 °C.

Japanese Patent 59 181 224 (Sumitomo Chemical Co.) describes an aqueous solution of interferons in which polar amino acids such as arginine, asparagine, glutamic acid, glutamine, histidine, lysine, serine and threonine or their sodium salts are used together with human serum albumin to stabilize the interferons.

International Patent Application WO 95/31213 (Applied Research Systems ARS Holding) describes a liquid formulation for interferon-β, which is stabilized by adding a polyol, preferably mannitol, and a non-reducing sugar or an amino acid. The formulation also contains a buffer (acetate buffer, pH 3.0 to 4.0) as well as human serum albumin. Although formulations having a pH value between 5 and 6 have shown an immediate loss of biological activity, the formulations preferred in this patent publication have sufficient stability at a pH value of 3.0 or 4.0. In this case, the stability is evaluated on the basis of the biological activity of the formulation but not the molecular integrity of the active ingredient.

European Patent Application EP 0 215 658 (Cetus Corp.) describes a formulation for recombinant interferon-β in which the biologically active compound is dissolved in an aqueous medium at a pH between 2 and 4 with the addition of stabilizers such as human serum albumin or human plasma protein fractions and optionally dextrose. Another patent application by Cetus Corp. (International Patent WO 89/05158) describes an interferon-β formulation which has a pH between 2 and 4 and uses as stabilizers either glycerol or polyethylene glycol polymers with an average molecular weight between 190 and 1600 daltons. Suitable buffer components include glycine, phosphoric acid and citric acid.

European Patent EP 0 217 645 (Cetus Corp.) describes pharmaceutical preparations containing IL-2 or interferon-β which are dissolved in a carrier medium at a pH of 7 to 8 and are stabilized with the addition of sodium laurate as the surface-active compound. In addition, SDS is also needed as an additional ionic surfactant compound to stabilize these preparations.

European Patent EP 0 270 799 (Cetus Oncology Corp.) describes a formulation for nonglycosylated recombinant interferon-β in an inert carrier medium based on water, containing nonionic polymer detergents as stabilizers.

European Patent Application EP 0 529 300 (Rentschler Biotechnologie GmbH) describes liquid interferon-β formulations which contain recombinant IFN-β in a concentration of 30 or 70 MU/mL plus sodium chloride and imidazole or sodium phosphate buffer at a pH of 7.5 (Example 3). These formulations are stable for four weeks at a storage temperature of 25 °C with respect to their biological activity. However, these combinations have the disadvantage that the concentration of interferon-β used in them ($\geq$ 30 MU/mL) is too high for practical applications. In addition, there is no mention in European Patent Application EP-A 0 529 300 that the stability of liquid interferon-β formulations is reduced by the addition of human serum albumin. On the contrary, the addition of human serum albumin is mentioned as preferred.

In addition to formulations of interferon-β, pharmaceutical forms of administration containing interferon-ϒ have also been described. European Patent 0 082 481 (Schering Corp.) discloses an aqueous formulation intended for freeze drying which contains human serum albumin in addition to a phosphate buffer. Alanine is mentioned as another optional ingredient. The pH value of the solution after reconstitution is between 7.0 and 7.4. Another patent application by Schering Corp. (International Patent WO 96/11018) discloses stable aqueous solutions of interferon-α which contain (at a pH between 4.5 and 7.1) chelating agents (NaEDTA or citric acid), a surfactant compound (polysorbate 80), an isotonizing agent (sodium chloride) as well as suitable preservatives such as methylparaben, propylparaben, *m*-cresol or phenol. The aqueous formulations disclosed there have proven to be stable (biological activity > 90% of the starting activity) with regard to their biological activity for six months at 25 °C (standard method of inhibition of the cytopathic effect (CPE) of a virus as described by W. P. Protzman in *J. Clinical Microbiology*, 1985, 22, pp. 596-599). However, a parallel determination of the protein content by HPLC shows a decline in content amounting to between 20.2 % (Table 3) and 32.5 % (Table 4) after only six months at 25 °C.

European Patent Application EP-A 0 736 303 (Hoffmann LaRoche AG) discloses aqueous interferon-ϒ compositions which contain, in addition to an interferon-α, a nonionic detergent, a buffer for adjusting the pH value to a range between 4.5 and 5.5, benzyl alcohol and an optional isotonizing agent. With an initial concentration of 18 MU interferon-αZ2e, a residual interferon content of 84.5 % is found in a determination by means of HPLC after three months of storage at 25 °C, but this value drops to 62.8 % when benzyl alcohol is omitted as the stabilizer.

European Patent Application EP-A 0 641 567 (Ciba Geigy AG) describes pharmaceutical compositions containing hybrid interferon-α and, as the stabilizer, a buffer at a pH between 3.0 and 5.0.

U.S. Patent 5,358,708 (Schering Corp.) describes aqueous formulations of interferon-α containing as the stabilizer methionine, histidine or mixtures thereof. After two weeks of storage of an interferon-α solution at 40 °C, a 20 % drop in the active ingredient content is found.

According to the prevailing standpoint today, the formulations of interferons listed above have numerous disadvantages, because the addition of human serum albumin should now be avoided for stabilization of proteins because of increased demands regarding safety from viral contamination due to blood donors. In addition, for a number of the formulations described above, it is also necessary to add amino acids and/or to perform freeze-drying. However, freeze-dried products are very complicated to manufacture and are expensive accordingly, and they require an additional step due to the need for reconstitution; however, this is often difficult to do for patients with restricted motor capacity. A number of formulations have a non-physiological pH value of less than 5.0. Although such values are not completely unconventional (see also S. Sweetans and N. J. Aders, *Journal of Pharmaceutical Sciences and Technology*, 1996, 50:330-342), painful irritation must be expected when these preparations are administered intramuscularly or subcutaneously. Although it is admissible to use surfactant compounds such as polysorbate 80 (according to Sweetana and Akers), however, a number of side effects have been described, especially in children

and neonates, casting doubt on the use of such additives. The toxicity of surfactant compounds is reported in a summary form by Attwood and Florence (*Surfactant Systems, Their Chemistry, Pharmacy and Biology,* Chapman and Hall; London, 1983). A review of the pharmacology of polysorbate 80 can be found in R. K. Varma et al. (*Arzneim.-Forsch./Drug Research*, 35, 1985, 804-808).

Because of the disadvantages mentioned above, an optimum formulation for interferon-β should combine the following properties:

-Preserving the biological activity over the entire storage period,

-Preserving the molecular integrity of the active ingredient molecule over the entire storage period,

-Liquid formulation, no expensive freeze drying or additional reconstitution,

-Avoiding high-risk additives such as human serum albumin or surfactant compounds (detergents),

-pH value in the neutral to weakly acidic range.

All requirements are met by the present invention, which is described in greater detail in the following section.

Surprising, a formulation has been discovered which ensures the molecular integrity of interferon-β in liquid form over a long period of time in a physiological pH range between 5 and 8, preferably from 5.5 to 8 without having to rely on the known additives of the state of the art.

Another aspect of the present invention is therefore a liquid pharmaceutical formulation which contains human interferon-β as an active ingredient in a concentration of up to 25 MU/mL and a buffer to establish a certain pH value, preferably between greater than 5.5 and 8, is free of human serum albumin, and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage for three months at 25 °C.

Another aspect of this invention is a liquid pharmaceutical formulation which contains human interferon-β as the active ingredient and a buffer for adjusting the pH to a value between 6 and 7.2, is free of human serum albumin and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage for three months at 25 °C.

Yet another aspect of this invention is a liquid pharmaceutical formulation which contains human IFN-β as the active ingredient, a buffer for adjusting a pH between 5 and 8, preferably between greater than 5.5 and 8, contains one or more amino acids and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage for three months at 25 °C.

The long-term stability of liquid pharmaceutical formulations was measured at 25 °C. A temperature of 25 °C was selected to accelerate degradation reactions on the one hand while on the other hand not causing any artefacts to be formed due to excessive temperatures. Suitable analytical methods for determining the stability of interferon data are described in the review article by J. Geigert (*J. Parent. Sci. Technol.* 43 (1989) 220-224) or M. C. Manning, K. Patel and R. T. Borchardt (*Pharm. Res.* 6 (1989), 903-918).

The biological activity was measured after the specified storage period by the standard method of inhibition of the cytopathic effect of a virus. A precise description of the test method used can be found in W. E. Stewart II (1981): *The Interferon System* (second enlarged edition), Springer Verlag, Vienna, New York; S. E. Grossberg et al. (1984), "Assay of Interferons," in: P. E. Came, W. A. Carter (eds.), *Interferons and Their Applications*, Springer Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 23-43.
After three months of storage at 25 °C, a formulation according to this invention will have a biological activity of at least 80 %, preferably at least 85 %, and especially preferably at least 90 % of the initial activity.
A formulation according to this invention will preferably have a biological activity of at least 80 % and preferably at least 85 % of the initial activity after storage for six months at 25 °C.
Even in the case of storage at a higher temperature, such as 37 °C, the formulations according to this invention have a surprisingly high long-term stability of the biological activity. Thus, after storage for one month at 37 °C, a biological activity of at least 70 % and preferably at least 80 % of the initial activity was found.
The liquid pharmaceutical formulations according to this invention are preferably free of human serum albumin and especially preferably free of human or animal polypeptides (apart from the active ingredient), especially serum proteins. In addition, it is preferable for the liquid pharmaceutical formulation according to this invention to be free of surfactant agents, and in particular free of ionic detergents and/or nonionic surfactants.
The formulations according to this invention contain as the active ingredient an interferon-β , i.e., a polypeptide which has the biological and/or immunological properties of natural human interferon-β and may be a naturally occurring or recombinant interferon-β . The formulation preferably contains a glycosylated interferon-β , especially preferably a recombinant interferon-β from CHO cells. The most preferred are interferon-β species such as those obtained from the cell line BiC 8622 (ECACC 87 04 03 01) and described in European Patent EP-B-0 287 075 and European Patent Application EP-A-0 529 300, for example.
The active ingredient is preferably present in the formulations according to this invention in a concentration of up to 25 MU/mL. However, a dosage in the range of 1 to 25 MU/mL is preferred, especially preferably from 3 to 20 MU/mL and most preferably from 3 to 10 MU/mL. These dosage ranges allow direct use without further dilution plus an especially good stability at elevated temperatures.
Another preferred feature of the liquid pharmaceutical formulation according to this invention is that it has chemical integrity after storage at 25 °C for three months and preferably for six months, i.e., it is stable with respect to peptide cleavage, oxidation and deglycosylation. The chemical integrity is measured by peptide mapping, Western Blot and glycosylation analysis. Compositions in which the interferon-β retains at least 85 %, preferably at least 90 % of its chemical integrity under the selected storage conditions are considered to be chemically stable in conjunction with the present invention.
Another preferred feature of the liquid pharmaceutical formulations according to this invention is their physical integrity after storage for three months at 25 °C and preferably for six months. The physical

integrity is measured by measuring the transmittance at 420 nm and by visual observation of the solutions. The solutions which are physically stable are those whose transmittance is more than 90 %, preferably more than 93 % under the selected storage conditions and in which no turbidity can be detected by visual observation.

Surprisingly, liquid formulations of interferon-β which are biologically, chemically and physically stable over a long period of time and are free of unwanted ingredients such as human serum albumin or surfactant agents can be made available through the present invention. The formulations according to this invention contain, in addition to the active ingredient, a buffer which is preferably present in a concentration of 10 mmol/L to 1 mol/L, especially preferably in a concentration of 20 mmol/L to 200 mmol/L, e.g., approx. 50 mmol/L to 100 mmol/L, and which serves to keep the pH of the formulation in the range of 5 to 8, preferably from more than 5.5 to 8 and even more preferably between 6 and 7.4. A pH between 6 and 7.2 is especially preferred, and a pH between 6.2 and 6.8 is most preferred, because this is where an especially high stability is achieved while maintaining molecular integrity. The buffer is selected from pharmaceutically acceptable buffers such as borate, succinate, L-malate, Tris, salicylate, glycylglycine, triethanolamine, isocitrate, maleate, phosphate, citrate and acetate buffers or mixtures thereof. The preferred buffers are phosphate, citrate and acetate buffers or mixtures thereof, especially preferably phosphate/citrate buffer.

In addition to the active ingredient and the buffer, the formulation according to this invention may also contain other physiologically safe additives such as additives to adapt the tonicity to that of blood or tissue, such as non-reducing sugars, sugar alcohols such as mannitol, sorbitol, xylitol or glycerol. In addition, one or more amino acids such as alanine, arginine, glycine, histidine and/or methionine may be added to the formulation according to this invention to further increase its chemical stability. Methionine is preferred here. The methionine concentration is preferably in the range of 0.1 to 4 mmol/L. A concentration of 2 mmol/L is especially preferred. In addition, the formulation may contain thickeners to increase the viscosity, e.g., for ophthalmological applications. Examples of suitable thickeners include ophthalmologically suitable polymers such as carbopol, methylcellulose, carboxymethylcellulose, etc.

In addition, the composition according to this invention may also contain preservatives. For ophthalmological purposes, thiomersal, for example, may be used in an amount of 0.001 % to 0.004 % (weight/volume).

In addition, this invention also relates to pharmaceutical preparations which contain a liquid formulation of interferon-β as described above. These pharmaceutical preparations are especially suitable for oral, parenteral or ophthalmological application. These formulations are preferably prepared in single doses of 1 to 25 MU IFN- β. In addition, this invention also relates to a method of producing such pharmaceutical preparations, wherein a formulation according to this invention plus optionally other necessary pharmaceutically additives is prepared and converted to a suitable form of administration.

The formulation according to this invention can be stored in suitable washed and sterilized glass vials (hydrolytic class 1) with pharmaceutically acceptable rubber stoppers.

In addition, formulations according to this invention may also be packaged aseptically in ready-to-use syringes or in cartridge ampoules for use in self-injection systems. The aqueous solutions may be freeze

dried (although this is not preferred) by adding other additives with which those skilled in the art are familiar, and are then available in liquid form after being reconstituted.
Liquid multiple dosage forms and eyedrop solutions and drop solutions for oral administration may be prepared by adding suitable preservatives.
The additional additives needed for production of the corresponding forms of administration are familiar to those skilled in the art.
Finally, this invention relates to a method of improving the stability of the liquid formulation which contains human interferon-β as the active ingredient and a buffer for adjusting the pH value in the range of 5 to 8, preferably from greater than 5.5 to 8, characterized in that a formulation without human serum albumin and/or with one or more amino acids is used. The improvement in stability includes an improvement in the long-term stability of the biological activity (*in vitro*), the chemical integrity and/or the physical integrity as described above.
In addition, this invention is illustrated by the following examples.

Examples

An interferon-β obtained from CHO cells was used in all the examples.

1. Long-term stability of liquid interferon-β formulations at 25 °C

The following formulations were tested:

Formulation 1: 50 mmol/L sodium citrate, pH 5.0

Formulation 2: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0, 15 mg/mL human serum albumin, 2 mmol/L methionine, 50 mg/mL glycerol

Formulation 3: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0, 50 mg/mL glycerol, 2 mmol methionine

Formulation 4: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0, 2 mmol/L methionine

Formulation 5: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0

Formulation 17: 70 mmol/L sodium citrate, 50 mmol/L sodium phosphate, 2 mmol/L methionine, pH 6.5

The formulations were diluted to a content of approx. 10 to 15 MU/mL (i.e., 10 to 15 × $10^6$ IU/mL). With the exception of formula 17 (see below), the formulations were placed in glass vials of hydrolytic class 1 (DIN 2R vials) which were sealed with conventional chlorobutyl rubber stoppers and stored at 25 °C for the stated period of time. The biological activity (*in vitro*) was determined by the method described by W. E. Stewart II (1981): *The Interferon System* (second enlarged edition), Springer Verlag: Vienna New York; S. E. Grossberg et al. (1984), "Assay of Interferons," in: P. E. Came, W. A. Carter (eds.), *Interferons and their Applications*, Springer Verlag: Berlin, Heidelberg, New York, Tokyo, pp. 23-43.

The results are shown in Tables 1 through 5. The notation "% (Ref)" refers to the relative biological activity based on the biological activity of a reference specimen for the stated period of time at -20 °C. The notation "% (0Mo)" refers to the percentage biological activity based on the initial value at 0 months.

Table 1 (Formulation 1):

| Months | Active ingredient content | | | |
|---|---|---|---|---|
| | | | Recovery (25 °C) | |
| | -20 °C | 25 °C | % (Ref) | % (0 Mo) |
| 0 | 11.0 | 11.0 | 100 | 100 |
| 1 | 10.0 | 9.8 | 98 | 89 |
| 2 | 9.7 | 11.0 | 113 | 100 |
| 3 | 10.0 | 10.6 | 106 | 96 |
| 4 | 10.3 | 9.5 | 92 | 86 |
| 5 | 9.5 | 9.7 | 102 | 88 |
| 6 | 10.5 | 10.2 | 97 | 93 |

Table 2 (Formulation 2):

| Months | Active ingredient content | | | |
|---|---|---|---|---|
| | | | Recovery (25°C) | |
| | -20 °C | 25 °C | % (Ref) | % (0 Mo) |
| 0 | 13.9 | 13.9 | 100 | 100 |
| 1 | 14.0 | 11.9 | 85 | 86 |
| 2 | 13.0 | 11.6 | 89 | 83 |
| 3 | 13.1 | 9.6 | 73 | 69 |
| 4 | 12.5 | 8.8 | 70 | 63 |
| 5 | 11.0 | 8.2 | 75 | 59 |
| 6 | 13.3 | 8.4 | 63 | 60 |

Table 3 (Formulation 3):

| Months | Active ingredient content | | | |
|---|---|---|---|---|
| | | | Recovery (25 °C) | |
| | -20 °C | 25 °C | % (Ref) | % (0 Mo) |
| 0 | 12.5 | 12.5 | 100 | 100 |
| 1 | 9.4 | 10.0 | 106 | 80 |
| 2 | 8.3 | 11.5 | 139 | 92 |
| 3 | 7.8 | 11.8 | 151 | 94.4 |
| 4 | 6.8 | 10.3 | 151 | 82.4 |
| 5 | 6.6 | 11.2 | 170 | 89.6 |
| 6 | 7.8 | 13.4 | 172 | 107.2 |

Table 4 (Formulation 4):

| Months | Active ingredient content | | | |
|---|---|---|---|---|
| | | | Recovery (25 °C) | |
| | -20 °C | 25 °C | % (Ref) | % (0 Mo) |
| 0 | 11.4 | 11.4 | 100 | 100 |
| 1 | 10.5 | 10.2 | 97 | 89 |
| 2 | 11.9 | 11.1 | 93 | 97 |
| 3 | 10.8 | 10.0 | 93 | 88 |
| 4 | 10.4 | 9.3 | 89 | 82 |
| 5 | 11.6 | 8.4 | 72 | 74 |
| 6 | 12.4 | 9.5 | 77 | 83 |

Table 5 (Formulation 5):

| Months | Active ingredient content | | | |
|---|---|---|---|---|
| | | | Recovery (25 °C) | |
| | -20 °C | 25 °C | % (Ref) | % (0 Mo) |
| 0 | 11.3 | 11.3 | 100 | 100 |
| 1 | 11.0 | 9.7 | 88 | 86 |
| 2 | 11.7 | 10.1 | 86 | 89 |
| 3 | 11.1 | 10.2 | 92 | 90 |
| 4 | 11.3 | 10.2 | 90 | 90 |
| 5 | 12.0 | 9.2 | 77 | 81 |
| 6 | 11.0 | 9.7 | 88 | 66 |

It can be seen from the above tables that formulations which do not contain any human serum albumin (formulations 1, 3, 4 and 5) surprisingly have a better stability than a formulation (formulation 2) which contains human serum albumin.

In the case of formulation 17 (see above), an interferon solution without human serum albumin was adjusted to an activity of 6 MU/0.5 mL under aseptic conditions. The clear, colorless solution was then filtered under sterile conditions and packaged in 0.5 mL amounts in pre-sterilized disposable syringes and sealed. The ready-to-use syringes were stored at 25 °C and were tested for clarity, pH and biological activity, yielding the following results:

| Storage in months | pH | Clarity (%) | MU/syringe | | Recovery (25 °C) | |
|---|---|---|---|---|---|---|
| | | | -20 °C | 25 °C | % (Ref) | % (0 Mo) |
| 0 | 6.5 | 99.5 | 6.3 | 6.3 | 100 | 100 |
| 3 | 6.5 | 99.1 | 5.6 | 6.1 | 108 | 97 |

2. Long term stability of liquid IFN-β formulations at 37 °C

The following formulations were tested in ready-to-use syringes:

Formulation 6: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0, 2 mmol/L methionine

Formulation 7: 50 mmol/L sodium citrate, pH 5.0, 18 mg/mL glycerol, 2 mmol/L methionine

Formulation 8: 50 mmol/L sodium citrate, pH 5.0, 18 mg/mL glycerol, 15 mg/mL human serum albumin, 2 mmol/L methionine

Formulation 9: 50 mmol/L sodium citrate, pH 6.0, 18 mg/mL glycerol, 2 mmol/L methionine

Formulation 10: 50 mmol/L sodium citrate, pH 6.5, 18 mg/mL glycerol, 2 mmol/L methionine

The formulations were tested in dosages of 3 MU per 0.5 mL (dosage strength 3), 6 MU per 0.5 mL (dosage strength 6) and 12 MU per mL (dosage strength 12).

The results are shown in the following Table 6.

Table 6.

| Storage in months | Dosage strength 3 | | | | | Dosage strength 6 | | | | | Dosage strength 12 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Formulation | | | | | Formulation | | | | | Formulation | | | | |
| | 6 | 7 | 8 | 9 | 10 | 6 | 7 | 8 | 9 | 10 | 6 | 7 | 8 | 9 | 10 |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 1 | 71 | 80 | 61 | 74 | 69 | 72 | 85 | 63 | 86 | 84 | 87 | 88 | 71 | 76 | 84 |
| 2 | 51 | 82 | 33 | 74 | 85 | 51 | 81 | 43 | 80 | 76 | 69 | 68 | 48 | 77 | 81 |
| 3 | 44 | 76 | 23 | 63 | 65 | 48 | 64 | 36 | 73 | 69 | 66 | 72 | 35 | 80 | 81 |
| 4 | 33 | 51 | 16 | 51 | 61 | 46 | 65 | 26 | 84 | -- | -- | 64 | 24 | 78 | 79 |

The results in Table 6 show that the formulations according to this invention without human serum albumin surprisingly have an improved stability at 37 °C.

3. Chemical stability at 25 °C

To investigate the chemical stability of liquid formulations of IFN-β , seven batches were formulated and stored at 25 °C. After storage for three months and six months, the protein was characterized by means of Lys-C mapping and a complete carbohydrate analysis. Special attention was devoted to the formation of methionine sulfoxide and to desialylation.

In addition to formulation 10 (see above), the following formulations were tested:

Formulation 11: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, 2 mmol/L methionine, pH 7.0 to 7.2

Formulation 12: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0 to 7.2

Formulation 13: 50 mmol/L sodium citrate, 18 mg/mL glycerol, 2 mmol/L methionine, pH 5.0 to 5.2

Formulation 14: 50 mmol/L sodium citrate, 18 mg/mL glycerol, pH 5.0 to 5.2

Formulation 15: 50 mmol/L sodium citrate, 15 mg/mL human serum albumin (medical grade), 18 mg/mL glycerol, 2 mmol/L methionine, pH 5.0 to 5.2

Formulation 16: 50 mmol/L sodium citrate, 15 mg/mL human serum albumin (medical grade), 18 mg/mL glycerol, pH 5.0 to 5.2 (comparison)

In all batches, the IFN-β content was between 10 and 11 MU/mL.

Test procedure

To perform the analysis, it was necessary to increase the concentration of the specimens. In addition, in the case of batches 15 and 16, the human serum albumin had to be removed. Therefore, the batches were applied to an anti-β chromatography column. The starting volume per batch was 32 mL. Batches 13 to 16 were neutralized by adding 2.1 mL 0.4 mol/L $Na_2HPO_4$ and 2.1 mL 0.4 mol/L $Na_2PO_4$ before performing anti-β chromatography.

For the immunoadsorption of interferon-β on a monoclonal antibody to interferon-β (BO2 Sepharose 6B, crosslinked, from Celltech), a C10 chromatography column (Pharmacia) was packed with 5 mL BO2 Sepharose and rinsed three times with, each time, 5-10 gel volumes of PBS, 0.1 mol/L sodium phosphate, pH 2.0 and PBS/1 mol/L KCl at a linear flow rate of 1.0 cm/min.

Approx. 32 mL of the solution containing interferon/HSA was applied at a linear flow rate of 0.5 cm/min. The column was washed with 10 gel volumes of PBS/1 mol/L KCl at a linear flow rate of 1 cm/min until the OD dropped to the base line. Elution was performed with approx. 1-2 gel volumes of 0.1 mol/L sodium phosphate, pH 2, at a linear flow rate of 1 cm/min. Interferon-β was obtained as a single peak in a high purity. This eluate was suitable for the subsequent protein characterization.

Performing the analysis

1. Lys-C mapping

With the endoproteinase enzyme Lys-C from Achromobacter (AP), interferon-β was split into 12 peptides at the C-terminal end of lysine under reducing conditions.

Fifty µL eluate from anti-β chromatography (12.5-50 ϒg interferon-β) was placed in an Eppendorf reaction vessel and mixed with 5 µL 2 mol/L Tris. To this was added endoproteinase from the Wako company in an enzyme/substrate ratio of 1:10 (endoproteinase-Lys-C solution in 50 mmol/L Tris/HCl, pH 9.0). The solution was mixed and incubated for two hours at 30 °C. Then 5 µL of 0.1 mol/L DTT was added to the batch.

The peptides were separated on a reversed phase column (Vydac C18, 300 Å, 5 µm, 2.1 mm) using an HP 1090 M series HPLC system with a diode array detector at 214 mm [sic; nm], using a gradient of A: 0.1% (v/v) TFA and B: 0.1 (v/v) TFA/70% (v/v) acetonitrile. The peptides were numbered consecutively in the order of their retention times and were assigned to the following sequences.

| Sequence ID No. | Peptide | Position | Sequence |
|---|---|---|---|
| 1 | AP1 | 109-115 | EDFTRGK |
| 2 | AP2 | 100-105 | TVLEEK |
| 3 | AP3 | 46-52 | QLQQFQK |
| 4 | AP4(ox) | 116-123 | LM(ox)SSLHLK |
| 5 | AP4 | 116-123 | LMSSLHLK |
| 6 | AP6(ox) | 34-45 | DRM(ox)NFDIPEEIK |
| 7 | AP5 | 124-134 | RYYGRILHYLK |
| 8 | AP6 | 34-45 | DRMNFDIPEEIK |
| 9 | AP7 | 20-33 | LLWQLNGRLEYCLK |
| 10 | AP8(ox) | 1-19 | M(ox)SYNLLGFLQRSSNFQCQK |
| 11 | AP8 | 1-19 | MSYNLLGFLORSSNFODQK |
| 12 | AP9 | 137-166 | EYSHCAWTIVRVEILRNFYRNRLTGYLAN |
| 13 | AP10(ox) | 53-99 | EDAALTIYEM(ox)LQNIFAIFRQDSSS |

| | | | |
|---|---|---|---|
| | | | TGWENETIVENLLANVYHQINHLK |
| 14 | AP10 | 53-99 | EDAALTIYEMLQNIFAIFRQDSSS<br>TGWNETIVENLLANVYHQINHLK |

Literature:

Utsumi et al. (1989). Characterization of four different mammalian-cell-derived recombinant human interferon-β1. *Eur. J. Biochem.* 181, 545-553.

Utsumi et al. (1988): Structural characterization of fibroblast human interferon-β1. *J. Interferon Res.* 8, 375-384.

Allen, G (1981): Laboratory techniques in biochemistry and molecular biology. *Sequencing of Proteins and Peptides*. Elsevier Verlag.

Castagnota et al. (1988). HPLC in protein sequence determinations. *J. Chromatography* 440, 213-251.

In the peptides designated as (ox), the amino acid methionine is present in the form of methionine sulfoxide. The quantification is based on the determination of the amount of the peak area of the oxidized peptide relative to the total area of the intact peptide and the oxidized peptide. The amounts of oxidized methionines are very low in fresh preparations of interferon-β. During storage, this amount increases to varying extents, depending on the storage conditions (buffer, pH, temperature, etc.). This change is not desirable, because it can contribute to the instability of the interferon-β molecule and it can have a significant influence on the *in vivo* properties.

The amount of oxidized peptides AP4(ox), AP6 (ox), AP8(ox) and AP10(ox) is thus an important criterion for evaluating the chemical integrity of the interferon-β molecule in a liquid formulation.

2. Carbohydrate determination

In the first step, the oligosaccharides were separated from the polypeptide and desalinated.

Approx. 0.7 mL of the eluate from anti-β chromatography was dialyzed against 500 mL dialysis buffer (0.05 mol/L sodium phosphate, 0.10 mol/L NaCl, pH 7.25) for 16 to 20 hours at room temperature while stirring lightly in a dialysis tube (6 mm diameter, Sigma No. D-9277). Then the tube was cut open at one end, and the contents were stripped into an Eppendorf reaction vessel. After dialysis, the volume of the specimen was 1 mL.

To the dialyzed specimen were added 20 µL Tween 20 (10%) and 15 µL N-glycosidase F solution (Boehringer Mannheim) by pipette. This mixture was incubated by for hours at 37 °C. After conclusion of the incubation, the mixture was centrifuged for 10 minutes at 10,000 rpm, filtered through a 0.45 µm

filter and then chromatographed and fractionated over a desalination column (HR 10/10 Pharmacia No. 17-0591-01) with an isocratic gradient (eluent A: distilled water) at a flow rate of 1.0 mL/min. The free oligosaccharides were detected at 206 nm.

In the second step, the oligosaccharides thus released were separated on an ion exchanger and differentiated according to the number of sialic acid groups.

The oligosaccharides contained in the eluate of the desalination column (approx. 2 mL) were bound to an anion exchanger (Mono Q HR 5/5, Pharmacia No. 17-0546-01). The asialo forms are found in the eluate. Monosialo, disialo and trisialo forms eluted with the help of a shallow NaCl gradient are found definitely separated one after the other in the order given here.

Eluent A: Milli-Q water

Eluent B: 0.10 mol/L NaCl

Gradient:

| | | |
|---|---|---|
| 0 min | 100 % A | 0 % B |
| 5 min | 100 % A | 0 % B |
| 25 min | 33 % A | 67 % B |
| 26 min | 100 % A | 0 % B |

Flow rate: 0.75 mL/min

Running time: 26 min (with regeneration 36 min)

Detection: UV 206 nm

Detection of the individual oligosaccharide fractions was performed by means of a UV detector at 206 nm. The quantitative calculation was performed by integration of the areas of the individual peaks.

The monosialo, disialo and trisialo oligosaccharides were then passed through a desalination column as described above.

Then in the third step, the charged oligosacchardes were converted to neutral oligosaccharides by hydrolytic cleavage of the terminal sialic acid groups under acidic pH conditions.

To do so, approx. 15 µL of each oligosaccharide fraction and 15 µL Milli-Q water were placed in a micro test tube, and 30 µL 10 mmol/L $H_2SO_4$ was added. Then the mixture was heated to 80 °C for 90 minutes.

Next, the test tube was centrifuged at 5000 rpm for one minute, and the batch was pipetted into a mini-vial. The carbohydrates, which were then neutral, were bound to weak anions and to an anion exchange column (CarboPac PA1 (4 × 250 mm) P/N 35391, Dionex) at an alkaline pH. Elution was performed with a gradient as follows:

Eluent A: NaOH 0.15 mol/L

Eluent B: NaOH 0.15 mol/L sodium acetate 0.10 mol/L

Eluent C: NaOH 0.15 mol/L sodium acetate 0.75 mol/L

Gradient:

| | | | |
|---|---|---|---|
| 0 min | 95 % A | 5 % B | 0 % C |
| 2.0 min | 95 % A | 5 % B | 0 % C |
| 3.0 min | 85% A | 15 % B | 0 % C |
| 4.0 min | 85 % A | 15 % B | 0 % C |
| 28.0 min | 37 % A | 63 % B | 0 % C |
| 28.1 min | 90 % A | 0 % B | 10 % C |
| 45.0 min | 20 % A | 0 % B | 80 % C |
| 45.1 min | 95 % A | 5 % B | 0 % C |
| 50.0 min | 95 % A | 5 % B | 0 % C |

Flow rate: 1.0 mL/min

Running time: 50 min

Detection: PAD

PAD (pulsed amperometric detection) was used to determine the oligosaccharides. In this method, the oligosaccharide molecule is oxidized electrochemically and the resulting electric current is measured. PAD is an extremely sensitive method which permits detection in the ng range with no problem. The starting signal on the detector (in mV) is directly proportional to the carbohydrate content. Quantification is based on integration of the peak areas.

The specimens were stored temporarily at –20 °C between deglycosylation and analysis.

Literature

Townsend (1988): High performance anion exchange chromatography of oligosaccharides. *Analytical Biochemistry* 174, 459-470.

Results

1. Lys-C mapping

Lys-C mapping of batches 11 through 16 did not reveal any difference in comparison with the initial value with regard to the retention time or qualification [sic; quantification] of the peptides.

Determination of the methionine sulfoxide content during liquid storage yielded the results shown in Table 7 (storage for three months) and Table 8 (storage for six months).

Table 7

| Identification | Amount of AP4ox | Amount of AP6ox | Amount of AP8ox | Amount of AP10ox |
|---|---|---|---|---|
| $t_0$ value | < 5 % | 7.6 % | LOD | LOD |
| Formulation 11 | 7.3 % | 10.5 % | LOD | LOD |
| Formulation 12 | < 5 % | 11.6 % | LOD | LOD |
| Formulation 13 | < 5 % | 7.3 % | LOD | LOD |
| Formulation 14 | < 5 % | 9.4 % | LOD | LOD |
| Formulation 15 | < 5 % | 8.6 % | LOD | LOD |
| Formulation 16 | < 5 % | 10.8 % | LOD | LOD |

(LOD = not detectable)

Table 8

| Identification | Amount of AP4ox | Amount of AP6ox | Amount of AP8ox | Amount of AP10ox |
|---|---|---|---|---|
| $t_0$ value | < 5 % | 7.6 % | LOD | LOD |
| Formulation 10 | 7.6 % | 8.9 % | LOD | LOD |
| Formulation 11 | 7.7 % | 9.6 % | LOD | LOD |
| Formulation 12 | 12.0 % | 13.7 % | LOD | LOD |
| Formulation 13 | 7.4 % | 8.7 % | LOD | LOD |
| Formulation 14 | 13.7 % | 15.7 % | LOD | LOD |
| Formulation 15 | 7.4 % | 7.9 % | LOD | LOD |
| Formulation 16 | 18.0 % | 17.0 % | LOD | LOD |

It can be seen from Table 7 that after three months of storage, batches 13 and 15, which contain methionine, have a lower methionine sulfoxide content in comparison with the methionine-free batches. After six months of storage, the influence of the added methionine in batches 11, 13 and 15 is more apparent. Only a very slight increase in methionine sulfoxide content can be detected there. The methionine sulfoxide content increases somewhat more in the batches that do not contain any methionine initially, but it remains less than 10 % in the sum of all the oxidized methionine contents relative to the total methionine content.

2. Carbohydrate determination

Tables 9a, 9b, 10a, 10b, 11a and 11b show the results of the carbohydrate determinations after three months of storage and after six months of storage.

On its amino acid chain, interferon-β-1a has a carbohydrate structure which is composed of a defined sequence of monosaccharides. Depending on the type of branching, these are called biantennary structures (having two arms), triantennary structures (three arms) and tetraantennary structures (four arms).

The carbohydrate structure is composed of the monosaccharides mannose, fucose, N-acetylglucosamine, galactose and sialic acid.

Sialic acid is in a special position in several regards:

- It is the only monosaccharide having a charged group (carboxyl group).
- It always occurs in the terminal position and the carbohydrate chain.
- It is split off much more easily by enzymes or by hydrolysis than the other monosaccharides.

- Although the structure of the neutral carbohydrate chain is very constant, there are great variations in the amount of sialic acid, depending on the cell structure and the interferon purification method, among other things.

Literature:

Kagawa et al., *J. Biol. Chem.* 263 (1988), 17508-17515; European Patent EP-A-0 529 300.

The sialo status (percentage amount of individual sialo structures) was investigated after three months of storage (Table 9a) and after six months of storage (Table 9b). A carbohydrate structure which does not have any sialic acid in terminal position is known as an asialo. A carbohydrate structure having one sialic acid group in terminal position is a monosialo. A carbohydrate structure having two sialic acid groups in terminal position is a disialo. A carbohydrate structure having three sialic acid groups in terminal position is a trisialo.

In addition, the antennarity (percentage amount of individual types of branching) was determined after three months of storage (Table 10a) and after six months of storage (Table 10b). A carbohydrate structure having branching and thus having two terminal galactoses is referred to as a biantennary structure. It may be occupied terminally with anything between two sialic acid groups and none. A carbohydrate structure having two branchings and thus terminal galactoses is referred to as a triantennary structure. It may be occupied terminally by zero to three sialic acids.

In addition, the degree of sialylation (percentage occupancy of terminal galactose groups with sialic acid) was also determined after three months of storage (Table 11a) and after six months of storage (Table 11b).

The results show that a slight but reproducible disialylation occurs in storage at a pH of 5. Storage at a pH of 7 does not affect the degree of sialylation.

The afuco component indicated in batches 15 and 16 presumably originates from foreign proteins from the added serum albumin, which was not separated completely by anti-β chromatography.

With regard to the antennarity, there is no measurable influence due to the liquid storage.

Table 9a

| Identification | Asialo | Monosialo | Disialo | Trisialo |
|---|---|---|---|---|
| $t_0$ value | < 3 | 13.4 | 73.4 | 12.1 |
| Formulation 11 | < 3 | 14.0 | 74.1 | 11.9 |
| Formulation 12 | < 3 | 12.5 | 74.9 | 11.6 |
| Formulation 13 | < 3 | 16.5 | 70.4 | 12.0 |
| Formulation 14 | < 3 | 16.6 | 71.1 | 11.1 |
| Formulation 15 | < 3 | 15.8 | 70.0 | 13.0 |
| Formulation 16 | < 3 | 15.1 | 72.0 | 11.9 |

Table 9b

| Identification | Asialo | Monosialo | Disialo | Trisialo |
|---|---|---|---|---|
| $t_0$ value | < 3 | 13.4 | 73.4 | 12.1 |
| Formulation 10 | < 3 | 13.9 | 70.2 | 15.3 |
| Formulation 11 | < 3 | 14.5 | 73.9 | 11.0 |
| Formulation 12 | < 3 | 14.0 | 72.4 | 13.5 |
| Formulation 13 | < 3 | 18.6 | 68.9 | 11.7 |
| Formulation 14 | < 3 | 19.0 | 69.4 | 10.7 |
| Formulation 15 | < 3 | 17.0 | 71.0 | 11.3 |
| Formulation 16 | < 3 | 16.1 | 71.5 | 12.4 |

Table 10a

| Identification | Biantennary | Triantennary 1 > 6 | Triantennary + 1 repeat |
|---|---|---|---|
| $t_0$ value | 74.4 | 18.1 | 3.7 |
| Formulation 11 | 72.9 | 18.7 | 3.7 |
| Formulation 12 | 76.9 | 17.0 | 2.7 |
| Formulation 13 | 74.7 | 18.0 | 3.1 |
| Formulation 14 | 75.9 | 17.3 | 2.9 |
| Formulation 15 | 75.2 (incl. 5 % afuco) | 18.0 | 3.3 |
| Formulation 16 | 75.9 (incl. 5 % afuco) | 17.8 | 3.0 |

Table 10b

| Identification | Biantennary | Triantennary 1 > 6 | Triantennary + 1 repeat |
|---|---|---|---|
| $t_0$ value | 74.4 | 18.1 | 3.7 |
| Formulation 10 | 71.4 | 19.3 | 4.0 |
| Formulation 11 | 73.0 | 18.7 | 3.3 |
| Formulation 12 | 72.3 | 19.7 | 3.4 |
| Formulation 13 | 72.4 | 19.2 | 3.4 |
| Formulation 14 | 74.2 | 18.7 | 3.2 |
| Formulation 15 | 73.0 | 18.7 | 2.8 |
| Formulation 16 | 74.3 (incl. 4 % afuco) | 19.7 | 3.2 |

Table 11a

| Identification | Degree of sialylation |
|---|---|
| $t_0$ value | 88.3 |
| Formulation 11 | 87.0 |
| Formulation 12 | 88.2 |
| Formulation 13 | 85.8 |
| Formulation 14 | 85.8 |
| Formulation 15 | 86.6 |
| Formulation 16 | 86.9 |

Table 11b

| Identification | Degree of sialylation |
|---|---|

| | |
|---|---|
| $t_0$ value | 88.3 |
| Formulation 10 | 87.5 |
| Formulation 11 | 86.6 |
| Formulation 12 | 87.7 |
| Formulation 13 | 84.1 |
| Formulation 14 | 84.3 |
| Formulation 15 | 85.7 |
| Formulation 16 | 86.5 |

Claims

1. A liquid formulation which contains human interferon-β as an active ingredient in a concentration of up to 25 MU/mL and contains a buffer for adjusting the pH value at 5 to 8, is free of human serum albumin and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage at 25 °C for three months.

2. The liquid formulation containing human interferon-β as the active ingredient and a buffer for adjusting the pH value at 6 to 7.2, is free of human serum albumin and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage at 25 °C for three months.

3. The liquid formulation which contains human interferon-β as the active ingredient, a buffer for adjusting the pH value at 5 to 8 and one or more amino acids, and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage for three months for 25 °C.

4. The formulation according to Claim 1, characterized in that it contains a glycosylated interferon-β.

5. The formulation according to Claim 2, characterized in that the interferon-β originates from CHO cells.

6. The formulation according to one of Claims 1 through 5, characterized in that it contains the buffer in a concentration of 10 mmol/L to 1 mol/L.

7. The formulation according to one of Claims 1 through 6, characterized in that it contains a buffer selected from the group consisting of phosphate buffers, citrate buffers and acetate buffers and mixtures thereof.

8. The formulation according to Claim 7, characterized in that it contains a phosphate/citrate buffer.

9. The formulation according to one of Claims 1 and 3 through 8, characterized in that it has a pH between 6 and 7.2.

10. The formulation according to Claim 3, characterized in that it is free of human serum albumin.

11. The formulation according to one of Claims 1 through 10, characterized in that it is free of human or animal polypeptides apart from the active ingredient.

12. The formulation according to one of Claims 1 through 11, characterized in that it is free of surface-active compounds.

13. The formulation according to one of Claims 1 through 12, characterized in that it has chemical integrity after storage for six months at 25 °C.

14. The formulation according to one of Claims 1 through 13, characterized in that it has physical integrity after storage for six months at 25 °C.

15. The formulation according to one of Claims 1, 2 and 4 through 14, characterized in that it also contains one or more amino acids.

16. The formulation according to Claim 3 or 15, characterized in that it contains methionine.

17. The formulation according to Claim 16, characterized in that the methionine is present in a concentration of 0.1 to 4 mmol/L.

18. The formulation according to one of Claims 1 through 17, characterized in that it also contains additives for adjusting the tonicity.

19. The formulation according to one of Claims 1 through 18, characterized in that it also contains thickeners to increase the viscosity.

20. The formulation according to one of Claims 1 through 19, characterized in that it also contains physiologically acceptable preservatives.

21. A pharmaceutical preparation, characterized in that it contains a liquid formulation according to one of Claims 1 through 20.

22. The pharmaceutical preparation according to Claim 21 for oral, parenteral or ophthalmologic application.

23. The pharmaceutical preparation according to Claim 21 or 22 with single doses of 1 to 25 MU.

24. A method of producing a pharmaceutical preparation according to one of Claims 21 through 23, characterized in that a formulation according to one of Claims 1 through 20 and optionally other pharmaceutically necessary additives is prepared and converted to a suitable form of administration.

25. The method of improving the stability of a liquid formulation containing human interferon-β as the active ingredient and a buffer for adjusting the pH value at 5 to 8, characterized in that a formulation which contains one or more amino acids and/or does not contain any human serum albumin is used.

26. The method according to Claim 25, characterized in that the improvement in stability includes an improvement in the long-term stability of the biological activity (*in vitro*), the chemical integrity and/or the physical integrity.

Sequence Protocol

(1) General information:

(i) Applicant

(A) Name: Dr. Rentschler Biotechnologie GmbH

(B) Address: Erwin Rentschler Strasse 21

(C) City: Laupheim

(E) Country: Germany

(F) Zip code: D-88471

(ii) Name of the invention: Liquid interferon-β formulations

(iii) Number of sequences: 14

(iv) Computer readable version:

(A) Data medium: floppy disk

(B) Computer: IBM PC compatible

(C) Operating system: PC-DOS/MS-DOS

(D) Software: PatentIn Release #1.0 version #1.30 (EPA)

(2) Information on sequence ID No.: 1

(i) Sequence characteristics:

(A) Length: 7 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 109-115

(xi) Sequence description: Sequence ID No. 1

Glu Asp Phe Thr Arg Gly Lys

1 5

(2) Information on sequence ID No.: 2:

(i) Sequence characteristics:

(A) Length: 6 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 100-109

(xi) Sequence description: Sequence ID No. 2

Thr Val Leu Glu Glu Lys

1 5

(2) Information on sequence ID No.: 3

(i) Sequence characteristics:

(A) Length: 7 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 46-52

(xi) Sequence description: Sequence ID No. 3

Gln Leu Gln Gln Phe Gln Lys

1 5

(2) Information on sequence ID No.: 4

(i) Sequence characteristics:

(A) Length: 8 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 116-123

(ix) Feature:

(A) Name/key: modified site

(B) Position: 2

(D) Other information: /product "Xaa = Met (oxidized)"

(xi) Sequence description: Sequence ID No. 4

Leu Xaa Ser Ser Leu His Leu Lyc

1 5

(2) Information on sequence ID No.: 5

(i) Sequence characteristics:

(A) Length: 8 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 116-123

(xi) Sequence description: Sequence ID No. 5

Leu Met Ser Ser Leu His Leu Lys

1 5

(2) Information on sequence ID No.: 6

(i) Sequence characteristics:

(A) Length: 12 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 34-45

(ix) Feature:

(A) Name/key: modified site

(B) Position: 3

(D) Other information: /product "Xaa = Met (oxidized)"

(xi) Sequence description: Sequence ID No. 6

Asp Arg Xaa Asn Phe Asp Ile Pro Glu Glu Ile Lys

1 5 10

(2) Information on sequence ID No.: 7

(i) Sequence characteristics:

(A) Length: 11 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 124-136

(xi) Sequence description: Sequence ID No. 7

Arg Tyr Tyr Gly Arg Ile Leu His Tyr Leu Lys

1 5 10

(2) Information on sequence ID No.: 8

(i) Sequence characteristics:

(A) Length: 12 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 34-45

(xi) Sequence description: Sequence ID No. 8

Asp Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Lys

1 5 10

(2) Information on sequence ID No.: 9

(i) Sequence characteristics:

(A) Length: 14 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 20-33

(xi) Sequence description: Sequence ID No. 9

Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu Tyr Cyc Leu Lys

1 5 10

(2) Information on sequence ID No.: 10

(i) Sequence characteristics:

(A) Length: 19 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 1-19

(ix) Feature:

(A) Name/key: modified site

(B) Position: 1

(D) Other information: /product "Xaa = Met (oxidized)"

(xi) Sequence description: Sequence ID No. 10

Xaa Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln

1 5 10 15

Cys Gln Lys

(2) Information on sequence ID No.: 11

(i) Sequence characteristics:

(A) Length: 19 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 1-19

(xi) Sequence description: Sequence ID No. 11

Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln

1 5 10 15

Cys Gln Lys

(2) Information on sequence ID No.: 12

(i) Sequence characteristics:

(A) Length: 30 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 137-166

(xi) Sequence description: Sequence ID No. 12

Glu Tyr Ser His Cys Ala Trp Thr Ile Val Arg Val Glu Ile Leu Arg

1 5 10 15

Asn Phe Tyr Phe Ile Asn Arg Leu Thr Gly Tyr Leu Arg Asn

20 25 30

(2) Information on sequence ID No.: 13

(i) Sequence characteristics:

(A) Length: 47 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 53-99

(ix) Feature:

(A) Name/key: modified site

(B) Position: 10

(D) Other information: /product "Xaa = Met (oxidized)"

(xi) Sequence description: Sequence ID No. 13

Glu Asp Ile Als Leu Thr Ile Tyr Glu Xaa Leu Gln Asn Ile Phe Als

1 5 10 15

Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn Glue Thr Ile Val

20 25 3

Glu Asn Leu Leu Ala Asn Val Tyr His Gln Ilc Asn His Leu Lys

35 40 45

(2) Information on sequence ID No.: 14

(i) Sequence characteristics:

(A) Length: 47 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 53-99

(xi) Sequence description: Sequence ID No. 14

Glu Asp Ala Ala Leu Thr Ile Tyr Glu Met Leu Gln Asn Ile Phe Ala
1 5 10 1

Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn Glu Thr Ile Val
20 25 30

Glu Asn Leu Leu Als Asn Val Tyr His Cln Ile Asn His Leu Lys
35 40 45

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(54) Title: LIQUID INTERFERON–$\beta$ FORMULATIONS

(54) Bezeichnung: FLÜSSIGE INTERFERON–$\beta$–FORMULIERUNGEN

(57) Abstract

The invention relates to liquid formulations of human interferon–$\beta$. The inventive formulations are characterised in that they have a buffer with a pH value in the slightly acidic to neutral range between 5 and 8, preferably between 5.5 and 8, and in that the interferon–$\beta$ is highly stable in solution, the molecular integrity being preserved.

(57) Zusammenfassung

Die voliegende Erfindung betrifft flüssige Formulierungen von humanem Interferon–$\beta$. Die Formulierungen sind dadurch gekennzeichnet, daß sie einen Puffer mit einem pH–Wert im schwach sauren bis neutralen Bereich zwischen 5 und 8, bevorzugt zwischen größer 5,5 und 8 aufweisen sowie eine hohe Stabilität des Interferon–$\beta$ in Lösung unter Beibehalt der molekularen Integrität gegeben ist.

*LEDIGLICH ZUR INFORMATION*

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# Flüssige Interferon-ß Formulierungen

## Beschreibung

Die vorliegende Erfindung betrifft flüssige Formulierungen von humanem Interferon-ß. Die Formulierungen sind dadurch gekennzeichnet, daß sie einen pH-Wert im schwach sauren bis neutralen Bereich zwischen 5 und 8 aufweisen sowie eine hohe Stabilität des Interferon-ß in Lösung unter Beibehalt der molekularen Integrität gegeben ist.

Natürlich vorkommende Interferone sind speziesspezifische Proteine, teilweise Glykoproteine, die durch unterschiedliche Zellen des Körpers nach Induktion mit Viren, doppelsträngiger RNA, anderen Polynukleotiden sowie Antigenen erzeugt werden. Interferone besitzen zahlreiche biologische Aktivitäten wie z.B. antivirale, antiproliferative sowie immunmodulatorische Eigenschaften. Es sind mindestens 3 unterschiedliche Typen humaner Interferone identifiziert worden, welche durch Leukozyten, Lymphozyten, Fibroblasten sowie Zellen des Immunsystems produziert werden und als $\alpha$-, ß-, $\gamma$-Interferone bezeichnet werden. Einzelne Interferontypen können weiterhin in zahlreiche Subtypen unterteilt werden.

Natives, menschliches Interferon-ß kann durch Superinduktion humaner Fibroblastenzellkulturen mit Poly-IC sowie anschließende Isolierung und Reinigung des Interferon ß durch chromatographische und elektrophoretische Techniken industriell hergestellt werden. Proteine oder Polypeptide, welche dem natürlichen Interferon-ß vergleichbare Eigenschaften aufweisen, können auch durch rekombinante DNA-Technologien hergestellt werden (EP-A-0 028 033; EP-A- 0 041 313; EP-A-0 070 906; EP-A-0 287 075; Chernajovsky et al. (1984) DNA 3, 297-308; McCormick et al. (1984) Mol. Cell. Biol. 4, 166-172). Dabei kann rekombinantes humanes Interferon-ß sowohl in eukaryontischen Zellen (z.B. CHO-Zellen) als auch von prokaryon-

tischen Zellen (z.B. E.coli) produziert werden. Die entsprechenden Interferone werden als Interferon-ß-1a bzw. Interferon-ß-1b bezeichnet. Im Gegensatz zu Interferon-ß-1b ist Interferon-ß-1a glykosiliert (Goodkin (1994) Lancet 344, 1057-1060).

Der therapeutische Einsatz von Interferon-ß setzt voraus, daß es in eine galenische Zubereitung gebracht wird, die das Protein über längere Zeit unter Erhaltung der molekularen Integrität lagerfähig macht. Interferon-ß ist instabil und unterliegt unterschiedlichen Abbaureaktionen. Hierzu gehören insbesondere die Spaltung von Peptidbindungen, Deamidierung, Oxidation des Methionins zu Methioninsulfid, Disulfidaustausch sowie Veränderungen der Zuckerseitenkette bis hin zur Deglycosilierung.

Aufgrund des therapeutischen Nutzens von Interferonen sind in den vergangenen Jahren eine Reihe von Formulierungen entwickelt worden, die jedoch alle gewisse Nachteile aufweisen. Das US-Patent Nr. 4,647,454 (Inter-Yeda Ltd.) beschreibt eine Formulierung von Fibroblasten Interferon-ß, die durch Zusatz von Polyvinylpyrrolidon (PVP) im stark sauren Bereich (pH 3,5) stabilisiert werden kann. Weitere bevorzugte Hilfsstoffe sind Mannitol, Humanserumalbumin sowie Acetatpuffer. Die Formulierung wird gefriergetrocknet und bei 4°C aufbewahrt.

Die japanische Patentschrift 59 181 224 (Sumitomo Chemical Co.) beschreibt eine wässrige Lösung von Interferonen, bei welcher polare Aminosäuren wie Arginin, Asparagin, Glutaminsäure, Glutamin, Histidin, Lysin, Serin sowie Threonin bzw. deren Natriumsalze zusammen mit Humanserumalbumin zur Stabilisierung der Interferone eingesetzt werden.

Die internationale Patentanmeldung WO 95/31213 (Applied Research Systems ARS Holding) beschreibt eine flüssige Formulierung für Interferon-ß, die durch Zusatz eines Polyols, bevorzugt Mannitol, und eines nichtreduzierenden Zuckers oder einer Aminosäure stabilisiert wird. Die

Formulierung enthält weiterhin einen Puffer (Acetatpuffer pH 3,0 bis 4,0) sowie Humanserumalbumin. Während Rezepturen mit einem pH-Wert zwischen 5 und 6 einen sofortigen Verlust an biologischer Aktivität zeigten, sind die in der Patentschrift bevorzugten Rezepturen bei pH-Werten von 3,0 sowie 4,0 hinreichend stabil. Die Aussage der Stabilität bezieht sich außerdem nur auf die biologische Aktivität der Formulierung, nicht aber auf die molekulare Integrität des Wirkstoffs.

Die europäische Patentanmeldung EP 0 215 658 (Cetus Corp.) beschreibt eine Formulierung für rekombinantes Interferon-ß, in welcher die biologisch aktive Verbindung in einem wässrigen Medium bei einem pH-Wert zwischen 2 und 4 unter Zusatz von Stabilisatoren wie Humanserumalbumin oder Humanplasmaproteinfraktionen sowie gegebenenfalls Dextrose gelöst wird. Eine weitere Patentanmeldung der Cetus Corp. (WO 89/05158) beschreibt eine Formulierung für Interferon-ß, die bei einem pH-Wert zwischen 2 und 4 als Stabilisatoren entweder Glycerin oder Polyethylenglycopolymere mit einem durchschnittlichen Molekulargewicht zwischen 190 bis 1.600 Dalton einsetzen. Als geeignete Pufferkomponenten werden Glycin, Phosphorsäure sowie Zitronensäure genannt.

Die europäische Patentanmeldung EP 0 217 645 (Cetus Corp.) beschreibt pharmazeutische Zubereitungen mit IL-2 oder Interferon-ß, die in einem Trägermedium bei pH 7 bis 8 gelöst und unter Zusatz von Natriumlaurat als oberflächenaktive Verbindung stabilisiert sind. Darüber hinaus wird zur Stabilisierung dieser Zubereitungen auch SDS als weitere ionische oberflächenaktive Verbindung benötigt.

Das europäische Patent EP 0 270 799 (Cetus Oncology Corp.) beschreibt eine Formulierung für nichtglycosiliertes rekombinantes Interferon-ß in einem inerten Trägermedium auf Wasserbasis, das als Stabilisator nichtionische polymere Detergenzien enthält.

Die europäische Patentanmeldung EP 0 529 300 (Rentschler Biotechnolgie GmbH) beschreibt flüssige Interferon-ß-Formulierungen, die eine Konzentration von 30 bzw. 70 MU/ml rekombinantes IFN-ß, Natriumchlorid und Imidazol- bzw. Natriumphosphatpuffer enthalten sowie einen pH-Wert von 7,5 aufweisen (Beispiel 3). Diese Formulierungen sind für 4 Wochen bei einer Lagertemperatur von 25°C hinsichtlich ihrer biologischen Aktivität stabil. Diese Zusammensetzungen haben jedoch den Nachteil, daß die verwendete Konzentration von Interferon-ß ($\geq$ 30 MU/ml) für praktische Anwendungen zu hoch ist. Darüber hinaus findet sich in EP-A-0 529 300 keinerlei Hinweis, daß durch Zusatz von Humanserumalbumin die Stabilität von flüssigen Interferon-ß Formulierungen verringert wird. Im Gegenteil wird der Zusatz von Humanserumalbumin als bevorzugt bezeichnet.

Neben Formulierungen für Interferon-ß sind auch pharmazeutische Darreichungsformen mit Interferon-$\alpha$ beschrieben. Die europäische Patentschrift 0 082 481 (Schering Corp.) offenbart eine zur Gefriertrocknung bestimmte wässrige Formulierung, die neben einem Phosphatpuffer und Glycin Humanserumalbumin enthält. Als weiterer optionaler Bestandteil wird Alanin genannt. Der pH-Wert der Lösung nach Rekonstitution liegt zwischen 7,0 und 7,4. Eine weitere Patentanmeldung der Schering Corp. (WO 96/11018) offenbart stabile wässrige Lösungen im Interferon-$\alpha$, die bei einem pH-Wert zwischen 4,5 und 7,1 Chelatbildner (NaEDTA oder Zitronensäure), eine oberflächenaktive Verbindung (Polysorbat 80), ein Isotonisierungsmittel (Natriumchlorid) sowie geeignete Konservierungsmittel wie Methylparaben, Propylparaben, m-Kresol oder Phenol beinhalten. Die offenbarten wässrigen Formulierungen erweisen sich bezüglich der biologischen Aktivität (Standardmethode der Hemmung des zytopatischen Effekts (CPE) eines Virus wie beschrieben bei W.P. Protzman in J. Clinical Microbiology, 1985, 22, S. 596-599) bei 25°C für 6 Monate als stabil (biologische Aktivität >90% der Ausgangsaktivität). Eine parallel durchgeführte Bestimmung des Proteingehalts mittels HPLC weist nach 6

Monaten bei 25°C jedoch bereits Gehaltsverluste zwischen 20,2 (Tab.3) oder 32,5% (Tab. 4) aus.

EP-A-0 736 303 (Hoffmann-LaRoche AG) offenbart wäßrige Interferon-α-Zusammensetzungen, die neben einem Interferon-α ein nichtionisches Detergens, einen Puffer zur Einstellung des pH-Bereiches zwischen 4,5 und 5,5, Benzylalkohol und gegebenenfalls ein isotonisierendes Mittel umfassen. Bei Bestimmung mittels HPLC wird nach dreimonatiger Lagerung bei 25°C und einer Ausgangskonzentration von 18 MU Interferon-α2a ein Restgehalt von 84,5 % ermittelt, bei Weglassen des Stabilisators Benzylalkohol sinkt dieser Wert auf 62,8 %.

EP-A-0 641 567 (Ciba Geigy AG) beschreibt pharmazeutische Zusammensetzungen, die Hybrid-Interferon-α und als Stabilisator einen Puffer mit einem pH-Wert zwischen 3.0 und 5.0 enthalten.

Das US-Patent 5,358,708 (Schering Corp.) beschreibt wässrige Formulierungen von Interferon-α, die als Stabilisator Methionin, Histidin oder Mischungen davon enthalten. Nach zweiwöchiger Lagerung einer Interferon-α Lösung bei 40°C wird eine Abnahme des Wirkstoffgehalts um 20 % gefunden.

Die oben aufgeführten Formulierungen für Interferone sind aus heutiger Sicht mit Nachteilen behaftet, da z.B. auf Zusatz von Humanserumalbumin zur Stabilisierung von Proteinen aus Gründen der gestiegenen Anforderungen an die Sicherheit vor Viruskontaminationen durch Blutspender verzichtet werden sollte. Desweiteren ist für eine Vielzahl der oben beschriebenen Formulierungen ein Zusatz von Aminosäuren und/oder eine Gefriertrocknung erforderlich. Gefriergetrocknete Produkte sind jedoch in ihrer Herstellung sehr aufwendig und entsprechend teuer und erfordern durch die Notwendigkeit zur Rekonstitution einen zusätzlichen Arbeitsschritt, der insbesondere für Patienten mit eingeschränkter Motorik oftmals nur sehr schwer zu

vollziehen ist. Eine Reihe von Rezepturen weisen unphysiologische pH-Werte unterhalb von 5,0 auf. Obschon derartige Werte nicht gänzlich unüblich sind (siehe auch S. Sweetana und N.J. Aders, Journal of Pharmaceutical Sciences and Technology, 1996, 50: 330-342), muß bei intramuskulärer oder subkutaner Applikation mit schmerzhaften Irritationen gerechnet werden. Die Verwendung von oberflächenaktiven Verbindungen, wie Polysorbat 80, ist entsprechend Sweetana und Akers zwar zulässig, es sind jedoch eine Reihe von Nebenwirkungen insbesondere auch bei Kindern und Neugeborenen beschrieben, die den Einsatz derartiger Hilfsstoffe in Frage stellen. Über die Toxizität von oberflächenaktiven Verbindungen wird zusammenfassend bei Attwood und Florence (Surfactant Systems, their Chemistry, Pharmacy and Biology, Chapman and Hall; London, 1983) berichtet. Eine Übersicht über die Pharmakologie von Polysorbat 80 befindet sich bei R.K. Varma et al. (Arzneim.-Forsch./ Drug Res. 35, 1985, 804-808).

Aufgrund der oben genannten Nachteile, sollte eine optimale Formulierung für Interferon-ß folgende Eigenschaften in sich vereinigen:

- Erhalt der biologischen Aktivität über den Lagerzeitraum,
- Erhalt der molekularen Integrität des Wirkstoffmoleküls über den Lagerzeitraum,
- Flüssige Formulierung, Verzicht auf eine teure Gefriertrocknung sowie eine zusätzliche Rekonstitution,
- Verzicht auf risikobehaftete Hilfsstoffe wie Humanserumalbumin oder oberflächenaktive Verbindungen (Detergenzien),
- pH-Wert im neutralen bis schwach sauren Bereich.

Sämtliche Forderungen werden durch die im nachfolgenden Abschnitt genauer beschriebene Erfindung erfüllt.

Überraschenderweise wurde eine Rezepturzusammensetzung gefunden, welche die molekulare Integrität von Interferon-ß in flüssiger Form über einen langen Zeitraum in einem physiologischen pH-Bereich zwischen 5 und 8, bevorzugt zwischen größer 5,5 und 8 sicherstellt, ohne auf die als nachteilig bekannten Hilfsstoffe des Standes der Technik zurückgreifen zu müssen.

Ein erster Aspekt der vorliegenden Erfindung ist daher eine flüssige pharmazeutische Formulierung, die humanes Interferon-ß als Wirkstoff in einer Konzentration bis zu 25 MU/ml und einen Puffer zur Einstellung eines pH-Werts von zwischen 5 und 8, bevorzugt zwischen größer 5,5 und 8 enthält, frei von Humanserumalbumin ist und eine Langzeitstabilität der biologischen Aktivität (in vitro) von mindestens 80% der Ausgangsaktivität nach Lagerung bei 25°C für 3 Monate aufweist.

Ein weiterer Aspekt der Erfindung ist eine flüssige pharmazeutische Formulierung, die humanes Interferon-ß als Wirkstoff und einen Puffer zur Einstellung eine pH-Werts zwischen 6 und 7,2 enthält, frei von Humanserumalbumin ist und eine Langzeitstabilität der biologischen Aktivität (in vitro) von mindestens 80% der Ausgangsaktivität nach Lagerung bei 25°C für 3 Monate aufweist.

Noch ein weiterer Aspekt der Erfindung ist eine flüssige pharmazeutische Formulierung, die humanes IFN-ß als Wirkstoff, einen Puffer zur Einstellung eines pH-Werts zwischen 5 und 8, bevorzugt zwischen größer 5,5 und 8 und eine oder mehrere Aminosäuren enthält und eine Langzeitstabilität der biologischen Aktivität (in vitro) von mindestens 80% der Ausgangsaktivität nach Lagerung bei 25°C für 3 Monate aufweist.

Die Messung der Langzeitstabilität von flüssigen pharmazeutischen Formulierungen erfolgte bei 25°C. Die Temperatur von 25°C wurde gewählt, um auf der einen Seite eine Beschleunigung von Abbaureaktionen zu bewirken, auf der anderen Seite jedoch keine durch überhöhte Temperaturen bewirk-

ten Artefakte hervorzurufen. Geeignete analytische Methoden zur Bestimmung der Stabilität von Interferon-ß sind in den Übersichtsartikeln von J. Geigert (J. Parent. Sci. Technol. 43 (1989), 220-224) oder M.C. Manning, K. Patel und R.T. Borchardt (Pharm. Res. 6 (1989), 903-918) beschrieben.

Die Messung der biologischen Aktivität nach der jeweils gewählten Aufbewahrungsdauer erfolgte durch die Standardmethode der Inhibierung des zytopathischen Effekts eines Virus. Eine genaue Beschreibung der verwendeten Testmethode findet sich bei Stewart, W.E. II (1981): The Interferon System (Second, enlarged Edition), Springer-Verlag: Wien, New York; Grossberg, S.E. et al. (1984), Assay of Interferons. In: Came, P.E., Carter W.A (eds) Interferons and their Applications, Springer-Verlag: Berlin, Heidelberg, New York, Tokyo, pp. 23-43. Eine erfindungsgemäße Formulierung weist nach dreimonatiger Aufbewahrung bei 25°C eine biologische Aktivität von mindestens 80%, vorzugsweise von mindestens 85% und besonders bevorzugt von mindestens 90% der Ausgangsaktivität auf.

Vorzugsweise besitzt eine erfindungsgemäße Formulierung nach sechsmonatiger Aufbewahrung bei 25°C eine biologische Aktivität von mindestens 80% und vorzugsweise von mindestens 85% der Ausgangsaktivität.

Auch bei einer Aufbewahrung bei höheren Temperaturen, z.B. 37°C, weisen die erfindungsgemäßen Formulierungen eine überraschend hohe Langzeitstabilität der biologischen Aktivität auf. So wird nach einer einmonatigen Aufbewahrung bei 37°C eine biologische Aktivität von mindestens 70% und vorzugsweise von mindestens 80% der Ausgangsaktivität gefunden.

Die erfindungsgemäßen flüssigen pharmazeutischen Formulierungen sind vorzugsweise frei von Humanserumalbumin und besonders bevorzugt - abgesehen vom Wirkstoff - frei von humanen oder tierischen Polypeptiden insbesondere von Serumproteinen. Weiterhin ist bevorzugt, daß die erfindungsgemäße flüssige pharmazeutische Formulierung frei von

oberflächenaktiven Mitteln, insbesondere frei von ionischen Detergenzien oder/und nichtionischen Tensiden ist.

Die erfindungsgemäßen Formulierungen enthalten als Wirkstoff ein Interferon-ß, d.h. ein Polypeptid, welches biologische oder/und immunologische Eigenschaften von natürlichem humanem Interferon-ß aufweist und ein natürlich vorkommendes oder rekombinantes Interferon-ß sein kann. Vorzugsweise enthält die Formulierung ein glykosiliertes Interferon-ß, besonders bevorzugt ein rekombinantes Interferon-ß aus CHO-Zellen. Am meisten bevorzugt werden Interferon-ß Spezies verwendet, wie sie aus der Zellinie BIC 8622 (ECACC 87 04 03 01) erhältlich sind und beispielsweise in EP-B-0 287 075 und EP-A-0 529 300 beschrieben sind.

Der Wirkstoff liegt in den erfindungsgemäßen Formulierungen vorzugsweise in einer Konzentration bis zu 25 MU/ml vor. Bevorzugt ist jedoch eine Dosierung im Bereich von 1 bis 25 MU/ml, besonders bevorzugt von 3 bis 20 MU/ml, am meisten bevorzugt 3 bis 10 MU/ml. Diese Dosierungsbereiche erlauben eine unmittelbare Anwendung ohne weitere Verdünnung in Verbindung mit einer besonders guten Stabilität bei erhöhter Temperatur.

Ein weiteres bevorzugtes Merkmal der erfindungsgemäßen flüssigen pharmazeutischen Formulierung ist, daß sie eine chemische Integrität nach Lagerung bei 25°C für 3 Monate und vorzugsweise für 6 Monate aufweist, d.h. daß sie beständig gegenüber Peptidspaltung, Oxidation und Deglykosilierung ist. Die Messung der chemischen Integrität erfolgt durch Peptidmapping, Westernblot sowie Glykosilierungsanalyse. Als chemisch stabil im Zusammenhang mit der vorliegenden Erfindung sind Zusammensetzungen zu betrachten, bei welchen das Interferon-ß im Anschluß an die Formulierung mindestens 85%, vorzugsweise mindestens 90% der chemischen Integrität bei den gewählten Lagerungsbedingungen beibehält.

Ein weiteres bevorzugtes Merkmal der erfindungsgemäßen flüssigen pharmazeutischen Formulierungen ist eine physikalische Integrität nach Lagerung bei 25°C für 3 Monate und vorzugsweise für 6 Monate. Dabei wird die physikalische Integrität durch Messung der Transmission bei 420 nm sowie durch visuelle Betrachtung der Lösungen gemessen. Als physikalisch stabil sind diejenigen Lösungen anzusehen, deren Transmission über 90%, vorzugsweise über 93% bei den gewählten Lagerungsbedingungen liegt, und bei welchen keine Trübung bei visueller Betrachtung festgestellt werden kann.

Durch die vorliegende Erfindung können überraschenderweise flüssige Formulierungen von Interferon-ß bereitgestellt werden, die über einen langen Zeitraum biologisch, chemisch und physikalisch stabil sowie frei von unerwünschten Inhaltsstoffen wie etwa Humanserumalbumin oder oberflächenaktiven Mitteln sind. Die erfindungsgemäßen Formulierungen enthalten neben dem Wirkstoff einen Puffer, der vorzugsweise in einer Konzentration von 10 mmol/l bis 1 mol/l, besonders bevorzugt in einer Konzentration von 20 mmol/l bis 200 mmol/l, z.B. ca. 50 mmol/l bis 100 mmol/l vorliegt und dazu dient, den pH-Wert der Formulierung im Bereich von 5 bis 8, bevorzugt von größer 5,5 bis 8 und stärker bevorzugt zwischen 6 und 7,4 zu halten. Besonders bevorzugt ist ein pH-Bereich zwischen 6 und 7,2 und am meisten bevorzugt zwischen 6,2 und 6,8, da hier eine besonders hohe Stabilität unter Beibehalt der molekularen Integrität erreicht wird. Der Puffer wird aus pharmazeutisch akzeptablen Puffern ausgewählt, z.B. Borat-, Succinat-, L-Malat-, TRIS-, Salicylat-, Glycylglycin-, Triethanolamin-, Isocitrat-, Maleat-, Phosphat-, Citrat- und Acetatpuffer oder Mischungen davon. Bevorzugt verwendet man Phosphat-, Citrat- und Acetatpuffer oder Mischungen davon, besonders bevorzugt Phosphat/Citratpuffer.

Die erfindungsgemäße Formulierung kann neben dem Wirkstoff und dem Puffer weitere physiologisch verträgliche Hilfsstoffe enthalten, beispielsweise Hilfsstoffe zur Anpassung der Tonizität an die Tonizität des Blutes

oder Gewebe, z.B. nichtreduzierende Zucker, Zuckeralkohole wie Mannit, Sorbit, Xylit oder Glycerin. Außerdem können der erfindungsgemäßen Formulierung eine oder mehrere Aminosäuren wie z.B. Alanin, Arginin, Glycin, Histidin oder/und Methionin zur weiteren Erhöhung der chemischen Stabilität zugesetzt werden. Bevorzugt ist hierbei Methionin. Die Konzentration von Methionin liegt vorzugsweise im Bereich von 0,1 bis 4 mmol/l. Besonders bevorzugt ist eine Konzentration vom 2 mmol/l. Weiterhin kann die Zusammensetzung Verdickungsmittel zur Viskositätserhöhung, z.B. für ophthalmologische Zwecke, enthalten. Beispiele für geeignete Verdickungsmittel sind ophthalmologisch geeignete Polymere, z.B. Carbopol, Methylcellulose, Carboxymethylcellulose etc.

Darüber hinaus kann die erfindungsgemäße Zusammensetzung auch Konservierungsmittel enthalten. Für ophthalmologische Zwecke kann beispielsweise Thiomersal in einer Menge von 0,001 bis 0,004% (Gewicht/Volumen) zum Einsatz kommen.

Die Erfindung betrifft weiterhin pharmazeutische Präparate, die eine flüssige Interferon-ß enthaltende Formulierung wie oben beschrieben enthalten. Diese pharmazeutischen Präparate sind insbesondere für die orale, parenterale oder ophthalmologische Applikation geeignet. Die Formulierungen liegen vorzugsweise in Einzeldosen von 1 bis 25 MU IFN-ß vor. Weiterhin betrifft die Erfindung ein Verfahren zur Herstellung derartiger pharmazeutischer Präparate, wobei man eine erfindungsgemäße Formulierung und gegebenenfalls weitere galenisch notwendige Hilfsstoffe zubereitet und in eine geeignete Darreichungsform bringt.

Die erfindungsgemäße Formulierung kann in geeigneten, gewaschenen sowie sterilisierten Glasvials (hydrolytische Klasse 1) mit pharmazeutisch akzeptablen Gummistopfen gelagert werden.

Desweiteren können erfindungsgemäße Formulierungen auch aseptisch in Fertigspritzen oder aber in Karpulen zum Einsatz in Selbstinjektionssystemen abgefüllt und eingesetzt werden. Die wässrigen Lösungen können - obwohl dies nicht bevorzugt ist - durch Zusatz weiterer, dem Fachmann bekannter Hilfsstoffe gefriergetrocknet werden und stehen dann nach Rekonstitution in flüssiger Form zur Verfügung.

Unter Zusatz von geeigneten Konservierungsmitteln können flüssige Mehrfachdosisarzneiformen sowie Augentropfenlösungen und Tropflösungen zur oralen Applikation hergestellt werden.

Die zur Herstellung der entsprechenden Darreichungsformen noch zusätzlich benötigten Hilfsstoffe sind dem Fachmann bekannt.

Schließlich betrifft die Erfindung ein Verfahren zur Verbesserung der Haltbarkeit einer flüssigen Formulierung, die humanes Interferon-ß als Wirkstoff und einen Puffer zur Einstellung eines pH-Werts von 5 bis 8, bevorzugt von größer 5,5 bis 8 enthält, dadurch gekennzeichnet, daß man eine Formulierung ohne Human-serumalbumin oder/und mit einer oder mehreren Aminosäuren verwendet. Die Verbesserung der Haltbarkeit umfaßt eine Verbesserung der Langzeitstabilität der biologischen Aktivität (in vitro), der chemischen Integrität oder/und der physikalischen Integrität wie vorstehend angegeben.

Weiterhin wird die Erfindung durch die nachfolgenden Beispiele erläutert.

**Beispiele**

In allen Beispielen wurde ein aus CHO-Zellen gewonnenes Interferon-ß verwendet.

1. Langzeitstabilität von flüssigen Interferon-ß Formulierungen bei 25°C

Es wurden folgende Formulierungen getestet:

Formulierung 1: 50 mmol/l Natriumcitrat pH 5,0

Formulierung 2: 50 mmol/l Natriumcitrat, 50 mmol/l, Natriumphosphat pH 7,0, 15 mg/ml Humanserumalbumin, 2 mmol/l Methionin, 50 mg/ml Glycerin

Formulierung 3: 50 mmol/l Natriumcitrat, 50 mmol/l Natriumphosphat pH 7,0, 50 mg/ml Glycerin, 2 mmol/l Methionin

Formulierung 4: 50 mmol/l Natriumcitrat, 50 mmol/l Natriumphosphat pH 7,0, 2 mmol/l Methionin

Formulierung 5: 50 mmol/l Natriumcitrat, 50 mmol/l Natriumphosphat pH 7,0

Formulierung 17: 70 mmol/l Natriumcitrat, 50 mmol/l Natriumphosphat, 2 mmol/l Methionin, pH 6,5

Die Formulierungen wurden auf einen Gehalt von ca. 10 bis 15 MU/ml (d.h. 10 bis 15 x $10^6$ I.E./ml) verdünnt.

Die Formulierungen wurden mit Ausnahme von Formulierung 17 (s.u.) in Glasvials der hydrolytischen Klasse 1 (DIN 2R Vials), die mit handelsüblichen Chlorbuthylgummistopfen verschlossen waren, bei 25°C für die angegebene Zeitdauer gelagert. Die Bestimmung der biologischen Aktivität (in vitro) erfolgte, wie beschrieben bei Stewart, W.E. II (1981): The Interferon System (Second, enlarged edition) Springer-Verlag: Wien, New York; Grossberg, S.E. et al. (1984) Assay of Interferons. In: Came, P.E., Carter W.A. (eds.) Interferons and their Applications, Springer-Verlag: Berlin, Heidelberg, New York, Tokyo, pp. 23-43.

Die Ergebnisse sind in den Tabellen 1 bis 5 dargestellt. Bei "% (Ref)" handelt es sich um die Angabe der biologischen Aktivität bezogen auf die biologische Aktivität einer bei -20°C für den angegebenen Zeitraum gelagerten Referenzprobe. Bei "% (0Mo)" handelt es sich um die prozentuale biologische Aktivität bezogen auf den Ausgangswert bei 0 Monaten.

Tabelle 1 (Formulierung 1):

| Monate | Wirkstoffgehalt | | | |
|---|---|---|---|---|
| | MU/mL | | Recovery (25°C) | |
| | -20°C | 25°C | % (Ref.) | % (0Mo.) |
| 0 | 11,0 | 11,0 | 100 | 100 |
| 1 | 10,0 | 9,8 | 98 | 89 |
| 2 | 9,7 | 11,0 | 113 | 100 |
| 3 | 10,0 | 10,6 | 106 | 96 |
| 4 | 10,3 | 9,5 | 92 | 86 |
| 5 | 9,5 | 9,7 | 102 | 88 |
| 6 | 10,5 | 10,2 | 97 | 93 |

Tabelle 2 (Formulierung 2):

| Monate | Wirkstoffgehalt | | | |
|---|---|---|---|---|
| | MU/mL | | Recovery (25°C) | |
| | -20°C | 25°C | % (Ref.) | % (0Mo.) |
| 0 | 13,9 | 13,9 | 100 | 100 |
| 1 | 14,0 | 11,9 | 85 | 86 |
| 2 | 13,0 | 11,6 | 89 | 83 |
| 3 | 13,1 | 9,6 | 73 | 69 |
| 4 | 12,5 | 8,8 | 70 | 63 |
| 5 | 11,0 | 8,2 | 75 | 59 |
| 6 | 13,3 | 8,4 | 63 | 60 |

Tabelle 3 (Formulierung 3):

| Monate | Wirkstoffgehalt | | | |
|---|---|---|---|---|
| | MU/mL | | Recovery (25°C) | |
| | -20°C | 25°C | % (Ref.) | % (0Mo.) |
| 0 | 12,5 | 12,5 | 100 | 100 |
| 1 | 9,4 | 10,0 | 106 | 80 |
| 2 | 8,3 | 11,5 | 139 | 92 |
| 3 | 7,8 | 11,8 | 151 | 94,4 |
| 4 | 6,8 | 10,3 | 151 | 82,4 |
| 5 | 6,6 | 11,2 | 170 | 89,6 |
| 6 | 7,8 | 13,4 | 172 | 107,2 |

Tabelle 4 (Formulierung 4):

| Monate | Wirkstoffgehalt | | | |
|---|---|---|---|---|
| | MU/mL | | Recovery (25°C) | |
| | -20°C | 25°C | % (Ref.) | % (0Mo.) |
| 0 | 11,4 | 11,4 | 100 | 100 |
| 1 | 10,5 | 10,2 | 97 | 89 |
| 2 | 11,9 | 11,1 | 93 | 97 |
| 3 | 10,6 | 10,0 | 93 | 88 |
| 4 | 10,4 | 9,3 | 89 | 82 |
| 5 | 11,6 | 8,4 | 72 | 74 |
| 6 | 12,4 | 9,5 | 77 | 83 |

Tabelle 5 (Formulierung 5):

| Monate | Wirkstoffgehalt | | | |
|---|---|---|---|---|
| | MU/mL | | Recovery (25°C) | |
| | -20°C | 25°C | % (Ref.) | % (0Mo.) |
| 0 | 11,3 | 11,3 | 100 | 100 |
| 1 | 11,0 | 9,7 | 88 | 86 |
| 2 | 11,7 | 10,1 | 86 | 89 |
| 3 | 11,1 | 10,2 | 92 | 90 |
| 4 | 11,3 | 10,2 | 90 | 90 |
| 5 | 12,0 | 9,2 | 77 | 81 |
| 6 | 11,0 | 9,7 | 88 | 86 |

Aus den obigen Tabellen ist ersichtlich, daß Formulierungen, die kein Humanserumalbumin enthalten (Formulierungen 1, 3, 4, 5), überraschenderweise eine bessere Stabilität als eine Humanserumalbumin enthaltende Formulierung (Formulierung 2) aufweisen.

Bei Formulierung 17 (s.o.) wurde eine Interferonlösung ohne Humanserumalbumin unter aseptischen Bedingungen auf eine Aktivität von 6 MU/0,5 ml eingestellt. Die farblose, klare Lösung wurde anschließend sterilfiltriert und zu je 0,5 ml in vorsterilisierten Einmalspritzen abgefüllt und verschlossen. Die Fertigspritzen wurden bei 25°C gelagert und auf Klarheit, pH-Wert sowie biologische Aktivität untersucht. Dabei ergaben sich folgende Resultate:

| Lagerung in Monaten | pH-Wert | Klarheit [%] | MU/Spritze | | Recovery (25°C) | |
|---|---|---|---|---|---|---|
| | | | -20°C | 25°C | %(Ref.) | %(0Mo.) |
| 0 | 6,5 | 99,5 | 6,3 | 6,3 | 100 | 100 |
| 3 | 6,5 | 99,1 | 5,6 | 6,1 | 108 | 97 |

2. Langzeitstabilität von flüssigen IFN-ß Formulierungen bei 37°C

Es wurden folgende Formulierungen in Fertigspritzen getestet:

Formulierung 6: 50 mmol/l Natriumcitrat, 50 mmol/l Natriumphosphat pH 7,0, 2 mmol/l Methionin

Formulierung 7: 50 mmol/l Natriumcitrat pH 5,0, 18 mg/ml Glycerin, 2 mmol/l Methionin

Formulierung 8: 50 mmol/l Natriumcitrat pH 5,0, 18 mg/ml Glycerin, 15 mg/ml Humanserumalbumin, 2 mmol/l Methionin

Formulierung 9: 50 mmol/l Natriumcitrat pH 6,0, 18 mg/ml Glycerin, 2 mmol/l Methionin

Formulierung 10: 50 mmol/l Natriumcitrat pH 6,5, 18 mg/ml Glycerin, 2 mmol/l Methionin

Die Formulierungen wurden in Dosisstärken von 3 MU pro 0,5 ml (Dosisstärke 3), 6 MU pro 0,5 ml (Dosisstärke 6) und 12 MU pro ml (Dosisstärke 12) getestet.

Die Ergebnisse sind in der nachfolgenden Tabelle 6 dargestellt.

Tabelle 6

| Lagerung in Monaten | Dosisstärke 3 | | | | | Dosisstärke 6 | | | | | Dosisstärke 12 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Formulierung | | | | | Formulierung | | | | | Formulierung | | | | |
| | 6 | 7 | 8 | 9 | 10 | 6 | 7 | 8 | 9 | 10 | 6 | 7 | 8 | 9 | 10 |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 1 | 71 | 80 | 61 | 74 | 69 | 72 | 85 | 63 | 86 | 84 | 87 | 88 | 71 | 76 | 84 |
| 2 | 51 | 82 | 33 | 74 | 85 | 61 | 81 | 43 | 80 | 76 | 69 | 88 | 48 | 77 | 81 |
| 3 | 44 | 76 | 23 | 63 | 65 | 48 | 64 | 36 | 73 | 69 | 66 | 72 | 35 | 80 | 81 |
| 4 | 33 | 51 | 16 | 61 | 61 | 46 | 65 | 26 | 84 | - | - | 64 | 24 | 78 | 79 |

Aus den Ergenissen von Tabelle 6 ist ersichtlich, daß die erfindungsgemäßen Formulierungen ohne Humanserumalbumin überraschenderweise eine verbesserte Stabilität bei 37°C aufweisen.

3. Chemische Stabilität bei 25°C

Um die chemische Stabilität flüssiger Formulierungen von IFN-ß zu untersuchen, wurden 7 Ansätze formuliert und bei 25°C eingelagert. Nach 3 bzw. 6 Monaten erfolgte eine Charakterisierung des Proteins mittels eines Lys-C-Mappings und einer kompletten Kohlenhydratanalytik. Ein spezielles Augenmerk wurde auf die Bildung von Methionin-Sulfoxid und die Desialyierung gelegt.

Außer Formulierung 10 (s.o.) wurden folgende Formulierungen getestet:

Formulierung 11: 50 mmol/l Natriumcitrat, 50 mmol/l Natriumphosphat, 2 mmol/l Methionin pH 7,0 bis 7,2

Formulierung 12: 50 mmol/l Natriumcitrat, 50 mmol/l Natriumphosphat pH 7,0 bis 7,2

Formulierung 13: 50 mmol/l Natriumcitrat, 18 mg/ml Glycerin, 2 mmol/l Methionin, pH 5,0 bis 5,2

Formulierung 14: 50 mmol/l Natriumcitrat, 18 mg/ml Glycerin, pH 5,0 bis 5,2

Formulierung 15: 50 mmol/l Natriumcitrat, 15 mg/ml Humanserumalbumin (Medical Grade), 18 mg/ml Glycerin, 2 mmol/l Methionin, pH 5,0 bis 5,2

Formulierung 16: 50 mmol/l Natriumcitrat, 15 mg/ml Humanserumalbumin (Medical Grade), 18 mg/ml Glycerin, pH 5,0 bis 5,2 (Vergleich)

Der Gehalt an IFN-ß lag bei allen Ansätzen zwischen 10 und 11 MU/ml.

Testdurchführung

Für die Durchführung der Analytik war eine Aufkonzentrierung der Proben notwendig. Zudem mußte bei den Ansätzen 15 und 16 das Humanserumalbumin entfernt werden. Deshalb wurden die Ansätze über eine Anti-ß-Chromatographiesäule gegeben. Das Ausgangsvolumen pro Ansatz betrug 32 ml. Die Ansätze 13 bis 16 wurden durch Zugabe von 2,1 ml 0,4 mol/l $Na_2HPO_4$ und 2,1 ml 0,4 mol/l $Na_3PO_4$ vor der Anti-ß-Chromatographie neutralisiert.

Für die Immunadsorption von Interferon-ß an einem monoklonalen Antikörper gegen Interferon-ß (B02 Sepharose 6B, cross linked von der Firma Celltech) wurde eine Chromatographiesäule C10 (Firma Pharmacia) mit 5 ml B02-Sepharose gepackt und 3 mal mit je 5-10 Gelvolumina PBS, 0,1 mol/l Natriumphosphat pH 2,0 und PBS/1 mol/l KCl, mit einer linearen Flußrate von 1,0 cm/min gespült.

Der Auftrag von ca. 32 ml der Interferon/HSA haltigen Lösung erfolgte mit einer linearen Flußrate von 0,5 cm/min.

Die Waschung erfolgte mit 10 Gelvolumina PBS/1 mol/l KCl mit einer linearen Flußrate von 1 cm/min bis zum Abfall der OD auf die Grundlinie. Die Elution erfolgte mit ca. 1-2 Gelvolumina 0,1 mol/l Natriumphosphat pH 2,0 mit einer linearen Flußrate von 1 cm/min. Interferon-ß wird dabei als einzelner Peak in hoher Reinheit erhalten. Dieses Eluat ist für die sich anschließende Proteincharakterisierung geeignet.

Durchführung der Analytik

1. Lys-C-Mapping

Mit dem Enzym Endoproteinase Lys-C aus Achromobacter (AP) wird Interferon-ß unter reduzierenden Bedingungen am C-terminalen Ende von Lysin in 12 Peptide gespalten.

In ein Eppendorf-Reaktionsgefäß wurden 50 µl Eluat aus der Anti-ß-Chromatographie (12,5-50 µg Interferon-ß) gegeben und mit 5 µl 2 mol/l TRIS versetzt. Dazu wurde Endoproteinase der Firma Wako in einem Enzym/Substratverhältnis von 1:10 zugegeben (Endoproteinase Lys-C-Lösung in 50 mmol/l TRIS/HCl, pH 9,0) Die Lösung wurde gemischt und bei 30°C 2 Stunden inkubiert. Danach erfolgte eine Zugabe von 5 µl 0,1 mol/l DTT zum Ansatz.

Die Auftrennung der Peptide erfolgte über eine Reversed Phase Säule (Vydac C18, 300 Å, 5 µm, 2,1 mm) an einer HPLC-Anlage HP 1090 M-Serie mit Diodenarraydetektor bei 214 mm, wobei ein Gradient aus A: 0,1% (v/v) TFA und B: 0,1% (v/v) TFA/70% (v/v) Acetonitril verwendet wurde. Die Peptide wurden in der Reihenfolge ihrer Retentionszeiten durchnummeriert und sind folgenden Sequenzen zugeordnet.

| SEQ. ID. NO | Peptid | Position | Sequenz |
|---|---|---|---|
| 1 | AP1 | 109-115 | EDFTRGK |
| 2 | AP2 | 100-105 | TVLEEK |
| 3 | AP3 | 46-52 | QLQQFQK |
| 4 | AP4(ox) | 116-123 | LM(ox)SSLHLK |
| 5 | AP4 | 116-123 | LMSSLHLK |
| 6 | AP6(ox) | 34-45 | DRM(ox)NFDIPEEIK |
| 7 | AP5 | 124-134 | RYYGRILHYLK |
| 8 | AP6 | 34-45 | DRMNFDIPEEIK |
| 9 | AP7 | 20-33 | LLWQLNGRLEYCLK |
| 10 | AP8(ox) | 1-19 | M(ox)SYNLLGFLQRSSNFQCQK |
| 11 | AP8 | 1-19 | MSYNLLGFLQRSSNFQCQK |
| 12 | AP9 | 137-166 | EYSHCAWTIVRVEILRNFYFINRLTGYLRN |
| 13 | AP10(ox) | 53-99 | EDAALTIYEM(ox)LQNIFAIFRQDSSS TGWNETIVENLLANVYHQINHLK |
| 14 | AP10 | 53-99 | EDAALTIYEMLQNIFAIFRQDSSS TGWNETIVENLLANVYHQINHLK |

Literatur:

Utsumi et al. (1989). Characterization of four different mammalian-cell-derived recombinant human interferon-ß1. Eur. J. Biochem. 181, 545-553.

Utsumi et al. (1988): Structural characterization of fibroblast human interferon-ß1. J. Interferon Res. 8, 375-384

Allen, G. (1981): Laboraory techniques in biochemistriy and molecular biology. Sequencing of proteins and peptides. Elsevier Verlag.

Castagnola et al. (1988): HPLC in Protein sequence determinations. J. Chromatography 440, 213-251.

In den mit (ox) bezeichneten Peptiden liegt die Aminosäure Methionin als Methioninsulfoxid vor. Die Quantifizierung beruht auf der Bestimmung des Anteils der Peakfläche des oxidierten Peptides zur Gesamtfläche aus intaktem Peptid und oxidiertem Peptid. Die Anteile an oxidierten Methioninen sind in frischen Präparationen von Interferon-ß sehr gering. Während der Lagerung nimmt dieser Anteil je nach Lagerbedingungen (Puffer, pH-Wert, Temperatur etc.) mehr oder weniger stark zu. Diese Veränderung ist nicht gewünscht, da sie zur Instabilität des Interferon-ß-Moleküls beiträgt bzw. die in vivo Eigenschaften signifikant beeinflussen kann.

Der Anteil der oxidierten Peptide AP4(ox), AP6(ox), AP8(ox) und AP10(ox) ist somit ein wichtiges Kriterium zur Bewertung der chemischen Integrität des Interferon-ß-Moleküls in einer flüssigen Formulierung.

2. Kohlenhydratbestimmung

Im ersten Schritt wurden die Oligosaccharide vom Polypeptid abgetrennt und entsalzt.

Etwa 0,7 ml des Eluats der Anti-ß-Chromatographie wurden in einem Dialyseschlauch (6 mm Duchmesser Sigma No. D-9277) gegen 500 ml Dialysepuffer (0,05 mol/l Natriumphosphat, 0,10 mol/l NaCl, pH 7,25) unter leichtem Rühren 16-20 Stunden bei Raumtemperatur dialysiert. Danach wurde der Schlauch an einem Ende aufgeschnitten und der Inhalt in ein Eppendorf-Reaktionsgefäß gestreift. Das Probevolumen betrug nach der Dialyse 1 ml.

Zu der dialysierten Probe wurden 20 µl Tween 20 (10%ig) und 15 µl N-Glycosidase-F-Lösung (Boehringer Mannheim) pipettiert. Dieses Gemisch

wurde 24 Stunden bei 37°C inkubiert. Nach Abschluß der Inkubation wurde bei 10.000 U/min 10 min zentrifugiert, über 0,45 µm filtiert und anschließend über eine Entsalzungssäule (HR 10/10 Pharmacia No. 17-0591-01) mit einem isokratischen Gradienten (Eluent A: destilliertes Wasser) mit einem Fluß von 1,0 ml/min chromatographiert und fraktioniert. Die Detektion der freien Oligosaccharide erfolgte bei 206 nm.

Im zweiten Schritt wurden die freigesetzten Oligosaccharide über einen Ionenaustauscher aufgetrennt nach der Anzahl ihrer Sialinsäurenreste differenziert.

Die im Eluat der Entsalzungssäule, ca. 2 ml, enthaltenen Oligosaccharide wurden an einen Anionenaustauscher (Mono Q HR 5/5, Pharmacia No. 17-0546-01) gebunden. Die Asialoformen finden sich im Durchlauf. Mit Hilfe eines flachen NaCl-Gradienten eluierten Monosialo-, Disialo- und Trisialoformen in der angegebenen Reihenfolge deutlich getrennt nacheinander.

Eluent A: Milli-Q-Wasser
Eluent B: 0,10 mol/l NaCl

Gradient

| | | |
|---|---|---|
| 0 min | 100% A | 0% B |
| 5 min | 100% A | 0% B |
| 25 min | 33% A | 67% B |
| 26 min | 100% A | 0% B |

Fluß: 0,75 ml/min
Laufzeit: 26 min (mit Regeneration 36 min)
Detektion: UV 206 nm

Die Detektion der einzelnen Oligosaccharidfraktionen erfolgte mittels eines UV-Detektors bei 206 nm. Die quantitative Berechnung wurde über die Integration der Flächen der einzelnen Peaks durchgeführt.

Die Oligosaccharidfraktionen Monosialo, Disialo und Trisialo wurden anschließend, wie oben beschrieben, über eine Entsalzungssäule geleitet.

Im dritten Schritt werden die geladenen Oligosaccharide in neutrale Oligosaccharide überführt, indem unter sauren pH-Bedingungen die endständigen Sialinsäurenreste hydrolytisch abgespalten wurden.

Dazu wurden von jeder Oligosaccharidfraktion ca. 15 µl plus 15 µl Milli Q Wasser in ein Mikroteströhrchen gegeben und 30 µl 10 mmol/l $H_2SO_4$ zugefügt. Anschließend wurde 90 min lang auf 80°C erhitzt.

Danach wurde 1 min bei 5000 U/min zentrifugiert und der Ansatz in ein Minivial pipettiert. Die jetzt neutralen Kohlenhydrate werden bei alkalischem pH-Wert zu schwachen Anionen und an eine Anionenaustauschersäule (CarboPac PA1 (4x250 mm) P/N 35391, Dionex) gebunden. Die Elution erfolgt mit einem Gradienten aus

Eluent A: NaOH 0,16 mol/l

Eluent B: NaOH 0,16 mol/l Na-Acetat 0,10 mol/l

Eluent C: NaOH 0,16 mol/l Na-Acetat 0,75 mol/l

Gradient:

| | | | |
|---|---|---|---|
| 0 min | 95% A | 5% B | 0% C |
| 2,0 min | 95% A | 5% B | 0% C |
| 3,0 min | 85% A | 15% B | 0% C |
| 4,0 min | 85% A | 15% B | 0% C |
| 28,0 min | 37% A | 63% B | 0% C |
| 28,1 min | 90% A | 0% B | 10% C |
| 45,0 min | 20% A | 0% B | 80% C |
| 45,1 min | 95% A | 5% B | 0% C |
| 50,0 min | 95% A | 5% B | 0% C |

Fluß: 1,0 ml/min

Laufzeit: 50 min

Detektion: PAD

Zur Bestimmung der Oligosaccharide wird die PAD (Pulsed Amperometric Detection) verwendet. Das Oligosaccharidmolekül wird elektrochemisch oxidiert und der dabei entstehende Strom gemessen. Die PAD zeichnet sich durch eine hohe Empfindlichkeit aus, so daß ein Nachweis im ng-Bereich ohne Schwierigkeiten möglich ist. Das Ausgangssignal am Detektor (in mV) ist der Menge an Kohlenydrat direkt proportional. Die Quantifizierung erfolgt über die Integration der Peakflächen.

Die Proben wurden zwischen der Deglykosilierung und der Analyse bei -20°C zwischengelagert.

Literatur:

Townsend (1988): High-performance anion-exchange chromatography of oligosaccharides. Analytical Biochemistry 174, 459-470.

Ergebnisse

1. Lys-C-Mapping

Das Lys-C-Mapping der Ansätze 11 bis 16 zeigte hinsichtlich der Retentionszeit und der Qualifizierung der Peptide keinen Unterschied zum Ausgangswert.

Die Bestimmung des Gehalts von Methioninsulfoxid während der Flüssiglagerung ergab die in den Tabellen 7 (Lagerung für 3 Monate) und 8 (Lagerung für 6 Monate) gezeigten Ergebnisse.

Tabelle 7

| Bezeichnung | Anteil AP4ox | Anteil AP6ox | Anteil AP8ox | Anteil AP10ox |
|---|---|---|---|---|
| to-Wert | < 5% | 7,6% | LOD | LOD |
| Formulierung 11 | 7,9% | 10,5% | LOD | LOD |
| Formulierung 12 | < 5% | 11,6% | LOD | LOD |
| Formulierung 13 | < 5% | 7,3% | LOD | LOD |
| Formulierung 14 | < 5% | 9,4% | LOD | LOD |
| Formulierung 15 | < 5% | 8,6% | LOD | LOD |
| Formulierung 16 | < 5% | 10,8% | LOD | LOD |

(LOD = nicht nachweisbar)

Tabelle 8

| Bezeichnung | Anteil AP4ox | Anteil AP6ox | Anteil AP8ox | Anteil AP10ox |
|---|---|---|---|---|
| to-Wert | < 5% | 7,6% | LOD | LOD |
| Formulierung 10 | 7,6% | 8,9% | LOD | LOD |
| Formulierung 11 | 7,7% | 9,5% | LOD | LOD |
| Formulierung 12 | 12,0% | 13,7% | LOD | LOD |
| Formulierung 13 | 7,4% | 8,7% | LOD | LOD |
| Formulierung 14 | 13,7% | 15,7% | LOD | LOD |
| Formulierung 15 | 7,4% | 7,9% | LOD | LOD |
| Formulierung 16 | 18,0% | 17,6% | LOD | LOD |

Aus Tabelle 7 ist ersichtlich, daß bei einer dreimonatigen Lagerung die methioninhaltigen Ansätze 13 und 15 gegenüber den methioninfreien Ansätzen einen geringeren Anteil an Methioninsulfoxid zeigen. Nach einer sechsmonatigen Lagerung wird der Einfluß des zugesetzten Methionins in den Ansätzen 11, 13 und 15 deutlicher. Dort ist nur eine sehr geringe Zunahme des Gehalts an Methioninsulfoxid nachweisbar. In den methioninfreien Ansätzen nimmt der Gehalt an Methioninsulfoxid etwas stärker zu, liegt aber in der Summe aller oxidierten Methioninanteile zum Gesamtgehalt an Methionin unter 10%.

2. Kohlenhydratbestimmung

In den Tabellen 9a, 9b, 10a, 10b, 11a und 11b sind die Ergebnisse der Kohlenhydratbestimmung nach drei bzw. nach 6 Monaten Lagerzeit angegeben.

Interferon-ß-1a besitzt an seiner Aminosäurenkette eine Kohlenhydratstruktur, die sich aus einer definierten Reihenfolge von Monosacchariden aufbaut. Je nach Verzweigungsart spricht man von biantennären (2 Arme), triantennären (3 Arme) und tetraantennären (4 Arme) Strukturen.

Die Kohlenhydratstruktur baut sich aus den Monosacchariden Mannose, Fucose, N-Acetylglucosamin, Galactose und Sialinsäure auf.

Dabei nimmt die Sialinsäure in mehrfacher Hinsicht eine Sonderstellung ein:

- Sie ist das einzige Monosaccharid mit einer geladenen Gruppe (Carboxylgruppe).
- Sie tritt immer endständig in der Kohlenhydratkette auf.
- Sie ist enzymatisch bzw. hydrolytisch wesentlich leichter abspaltbar als die restlichen Monosaccharide.
- Während die neutrale Kohlenhydratkette in ihrer Struktur sehr konstant ist, treten beim Anteil der Sialinsäure große Schwankungen auf, die unter anderem von der Zellkultur und dem Aufreinigungsverfahren des Interferons abhängig sind.

Literatur:

Kagawa et al., J. Biol. Chem. 263 (1988), 17508-17515; EP-A-0 529 300.

Es wurde der Sialostatus (prozentualer Anteil einzelner Sialostrukturen) nach dreimonatiger Lagerung (Tabelle 9a) bzw. sechsmonatiger Lagerung (Tabelle 9b) untersucht. Eine Kohlenhydratstruktur, die endständig keine Sialinsäure enthält, wird als Asialo bezeichnet. Eine Kohlenhydratstruktur die endständig eine Sialinsäure enthält, wird als Monosialo bezeichnet. Eine Kohlenhydratstruktur, die endständig zwei Sialinsäuren enthält, wird als Disialo bezeichnet. Eine Kohlenhydratstruktur, die endständig drei Sialinsäuren enthält, wird als Trisialo bezeichnet.

Weiterhin wurde die Antennärität (prozentualer Anteil einzelner Verzweigungsarten) nach dreimonatiger Lagerung (Tabelle 10a) bzw. nach sechsmonatiger Lagerung (Tabelle 10b) bestimmt. Eine Kohlenhydratstruktur mit einer Verzweigung und damit zwei endständigen Galactosen wird als biantennär bezeichnet. Sie kann terminal mit null bis zwei Sialinsäuren besetzt sein. Eine Kohlenhydratstruktur mit zwei Verzweigungen und damit drei endständigen Galactosen wird als triantennär bezeichnet. Sie kann terminal mit null bis drei Sialinsäuren besetzt sein.

Außerdem wurde der Sialylierungsgrad (prozentuale Belegung terminaler Galactosereste mit Sialinsäure) nach dreimonatiger Lagerung (Tabelle 11a) bzw. nach sechsmonatiger Lagerung (Tabelle 11b) untersucht.

Aus den Ergebnissen ist ersichtlich, daß durch die Lagerung bei pH 5 eine geringe, aber reproduzierbare Desialylierung stattfindet. Eine Lagerung bei pH 7 beeinflußt den Sialylierungsgrad nicht.

Der in den Ansätzen 15 und 16 angegebene afuco-Anteil stammt vermutlich von Fremdproteinen aus dem zugesetzten Humanserumalbumin, die durch die Anti-ß-Chromatographie nicht quantitativ abgetrennt wurden.

Bezüglich der Antennärität erfolgt kein meßbarer Einfluß durch die Flüssiglagerung.

Tabelle 9a

| Bezeichnung | Asialo | Monoasialo | Disialo | Trisialo |
|---|---|---|---|---|
| to-Wert | < 3 | 13,4 | 73,4 | 12,1 |
| Formulierung 11 | < 3 | 14,0 | 74,1 | 11,9 |
| Formulierung 12 | < 3 | 12,6 | 74,9 | 11,6 |
| Formulierung 13 | < 3 | 16,5 | 70,4 | 12,0 |
| Formulierung 14 | < 3 | 16,6 | 71,1 | 11,1 |
| Formulierung 15 | < 3 | 15,8 | 70,0 | 13,0 |
| Formulierung 16 | < 3 | 15,1 | 72,0 | 11,9 |

Tabelle 9b

| Bezeichnung | Asialo | Monosialo | Disialo | Trisialo |
|---|---|---|---|---|
| to-Wert | < 3 | 13,4 | 70,4 | 12,1 |
| Formulierung 10 | < 3 | 13,9 | 70,2 | 15,3 |
| Formulierung 11 | < 3 | 14,5 | 73,9 | 11,6 |
| Formulierung 12 | < 3 | 14,0 | 72,4 | 13,6 |
| Formulierung 13 | < 3 | 16,5 | 68,9 | 11,7 |
| Formulierung 14 | < 3 | 19,0 | 69,4 | 10,7 |
| Formulierung 15 | < 3 | 17,0 | 71,0 | 11,3 |
| Formulierung 16 | < 3 | 16,1 | 71,5 | 12,4 |

Tabelle 10a

| Bezeichnung | Biantennär | Triantennär 1->6 | Triantennär + 1 repeat |
|---|---|---|---|
| to-Wert | 74,4 | 18,1 | 3,7 |
| Formulierung 11 | 72,9 | 18,7 | 3,7 |
| Formulierung 12 | 76,9 | 17,0 | 2,7 |
| Formulierung 13 | 74,7 | 18,0 | 3,1 |
| Formulierung 14 | 75,9 | 17,3 | 2,9 |
| Formulierung 15 | 76,2 (inkl. 5% afuco) | 18,0 | 3,3 |
| Formulierung 16 | 76,9 (inkl. 5% afuco) | 17,8 | 3,0 |

Tabelle 10b

| Bezeichnung | Biantennär | Triantennär 1->6 | Triantennär + 1 repeat |
|---|---|---|---|
| to-Wert | 74,4 | 18,7 | 3,7 |
| Formulierung 10 | 71,4 | 19,3 | 4,0 |
| Formulierung 11 | 73,0 | 18,7 | 3,3 |
| Formulierung 12 | 72,3 | 19,7 | 3,4 |
| Formulierung 13 | 72,4 | 18,7 | 3,4 |
| Formulierung 14 | 74,2 | 18,7 | 3,2 |
| Formulierung 15 | 73,0 | 18,7 | 2,8 |
| Formulierung 16 | 74,3 (inkl. 4% afuco) | 19,7 | 3,2 |

Tabelle 11a

| Bezeichnung | Sialylierungsgrad |
|---|---|
| to-Wert | 88,3 |
| Formulierung 11 | 87,0 |
| Formulierung 12 | 88,2 |
| Formulierung 13 | 85,8 |
| Formulierung 14 | 85,8 |
| Formulierung 15 | 86,6 |
| Formulierung 16 | 86,9 |

Tabelle 11b

| Bezeichnung | Sialylierungsgrad |
|---|---|
| to-Wert | 88,3 |
| Formulierung 10 | 87,5 |
| Formulierung 11 | 86,6 |
| Formulierung 11 | 87,7 |
| Formulierung 13 | 84,1 |
| Formulierung 14 | 84,3 |
| Formulierung 15 | 85,7 |
| Formulierung 16 | 86,5 |

6. Formulierung nach einem der Ansprüche 1 bis 5,
**dadurch gekennzeichnet,**
daß sie den Puffer in einer Konzentration von 10 mmol/l bis 1 mol/l enthält.

7. Formulierung nach einem der Ansprüche 1 bis 6,
**dadurch gekennzeichnet,**
daß sie einen Puffer ausgewählt aus der Gruppe bestehend aus Phosphat-, Citrat- und Acetatpuffern und Mischungen davon enthält.

8. Formulierung nach Anspruch 7,
**dadurch gekennzeichnet,**
daß sie einen Phosphat/Citratpuffer enthält.

9. Formulierung nach einem der Ansprüche 1 und 3 bis 8,
**dadurch gekennzeichnet,**
daß sie einen pH-Wert zwischen 6 und 7,2 aufweist.

10. Formulierung nach Anspruch 3,
**dadurch gekennzeichnet,**
daß sie frei von Humanserumalbumin ist.

11. Formulierung nach einem der Ansprüche 1 bis 10,
**dadurch gekennzeichnet,**
daß sie abgesehen vom Wirkstoff frei von humanen oder tierischen Polypeptiden ist.

12. Formulierung nach einem der Ansprüche 1 bis 11,
**dadurch gekennzeichnet,**
daß sie frei von oberflächenaktiven Verbindungen ist.

13. Formulierung nach einem der Ansprüche 1 bis 12,
**dadurch gekennzeichnet,**
daß sie eine chemische Integrität nach Lagerung bei 25°C für 6 Monate aufweist.

14. Formulierung nach einem der Ansprüche 1 bis 13,
**dadurch gekennzeichnet,**
daß sie eine physikalische Integrität nach Lagerung bei 25°C für 6 Monate aufweist.

15. Formulierung nach einem der Ansprüche 1, 2 und 4 bis 14,
**dadurch gekennzeichnet,**
daß sie weiterhin eine oder mehrere Aminosäuren enthält.

16. Formulierung nach Anspruch 3 oder 15,
**dadurch gekennzeichnet,**
daß sie Methionin enthält.

17. Formulierung nach Anspruch 16,
**dadurch gekennzeichnet,**
daß das Methionin in einer Konzentration von 0,1 bis 4 mmol/l vorliegt.

18. Formulierung nach einem der Ansprüche 1 bis 17,
**dadurch gekennzeichnet,**
daß sie weiterhin Hilfsstoffe zur Einstellung der Tonizität enthält.

19. Formulierung nach einem der Ansprüche 1 bis 18,
**dadurch gekennzeichnet,**
daß sie weiterhin Verdickungsmittel zur Viskositätserhöhung enthält.

20. Formulierung nach einem der Ansprüche 1 bis 19,
**dadurch gekennzeichnet,**
daß sie weiterhin physiologisch verträgliche Konservierungsmittel enthält.

21. Pharmazeutisches Präparat,
**dadurch gekennzeichnet,**
daß es eine flüssige Formulierung nach einem der Ansprüche 1 bis 20 enthält.

22. Pharmazeutisches Präparat nach Anspruch 21 zur oralen, parenteralen oder ophthalmologischen Applikation.

23. Pharmazeutisches Präparat nach Anspruch 21 oder 22 mit Einzeldosen von 1 bis 25 MU.

24. Verfahren zur Herstellung eines pharmazeutischen Präparats nach einem der Ansprüche 21 bis 23,
**dadurch gekennzeichnet,**
daß man eine Formulierung nach einem der Ansprüche 1 bis 20 und gegebenenfalls weitere galenisch notwendige Hilfsstoffe zubereitet und in eine geeignete Darreichungsform bringt.

25. Verfahren zur Verbesserung der Haltbarkeit einer flüssigen Formulierung, die humanes Interferon-ß als Wirkstoff und einen Puffer zur Einstellung eines pH-Werts von 5 bis 8 enthält,
**dadurch gekennzeichnet,**
daß man eine Formulierung ohne Humanserumalbumin oder/und mit einer oder mehreren Aminosäuren verwendet.

26. Verfahren nach Anspruch 25,
**dadurch gekennzeichnet,**
daß die Verbesserung der Haltbarkeit eine Verbesserung der Langzeitstabilität der biologischen Aktivität (in vitro), der chemischen Integrität oder/und der physikalischen Integrität umfaßt.

SEQUENZPROTOKOLL

(1) ALLGEMEINE ANGABEN:

(i) ANMELDER:
(A) NAME: Dr.Rentschler Biotechnologie GmbH
(B) STRASSE: Erwin-Rentschler-Str. 21
(C) ORT: Laupheim
(E) LAND: Deutschland
(F) POSTLEITZAHL: D-88471

(ii) BEZEICHNUNG DER ERFINDUNG: Flüssige Interferon-ß Formulierungen

(iii) ANZAHL DER SEQUENZEN: 14

(iv) COMPUTER-LESBARE FASSUNG:
(A) DATENTRÄGER: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) BETRIEBSSYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPA)

(2) ANGABEN ZU SEQ ID NO: 1:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 7 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 109-115

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 1:

Glu Asp Phe Thr Arg Gly Lys
1 5

(2) ANGABEN ZU SEQ ID NO: 2:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 6 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 100-105

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 2:

Thr Val Leu Glu Glu Lys
1 5

(2) ANGABEN ZU SEQ ID NO: 3:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 7 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 46-52

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 3:

Gln Leu Gln Gln Phe Gln Lys
1 5

(2) ANGABEN ZU SEQ ID NO: 4:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 8 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 116-123

(ix) MERKMAL:
(A) NAME/SCHLÜSSEL: Modified-site
(B) LAGE:2
(D) SONSTIGE ANGABEN:/product= "Xaa = Met(oxidiert)"

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 4:

Leu Xaa Ser Ser Leu His Leu Lys
1 5

(2) ANGABEN ZU SEQ ID NO: 5:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 8 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 116-123

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 5:

Leu Met Ser Ser Leu His Leu Lys
1 5

(2) ANGABEN ZU SEQ ID NO: 6:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 12 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 34-45

(ix) MERKMAL:
(A) NAME/SCHLÜSSEL: Modified-site
(B) LAGE:3
(D) SONSTIGE ANGABEN:/product= "Xaa = Met(oxidiert)"

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 6:

Asp Arg Xaa Asn Phe Asp Ile Pro Glu Glu Ile Lys
1 5 10

(2) ANGABEN ZU SEQ ID NO: 7:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 11 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 124-134

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 7:

Arg Tyr Tyr Gly Arg Ile Leu His Tyr Leu Lys
1 5 10

(2) ANGABEN ZU SEQ ID NO: 8:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 12 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 34-45

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 8:

Asp Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Lys
1 5 10

(2) ANGABEN ZU SEQ ID NO: 9:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 14 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 20-33

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 9:

Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu Tyr Cys Leu Lys
1 5 10

(2) ANGABEN ZU SEQ ID NO: 10:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 19 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 1-19

(ix) MERKMAL:
(A) NAME/SCHLÜSSEL: Modified-site
(B) LAGE:1
(D) SONSTIGE ANGABEN:/product= "Xaa = Met(oxidiert)"

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 10:

Xaa Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln
1 5 10 15

Cys Gln Lys

(2) ANGABEN ZU SEQ ID NO: 11:

(i) SEQUENZKENNZEICHEN:

(A) LÄNGE: 19 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 1-19

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 11:

Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln
1 5 10 15

Cys Gln Lys

(2) ANGABEN ZU SEQ ID NO: 12:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 30 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 137-166

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 12:

Glu Tyr Ser His Cys Ala Trp Thr Ile Val Arg Val Glu Ile Leu Arg
1 5 10 15

Asn Phe Tyr Phe Ile Asn Arg Leu Thr Gly Tyr Leu Arg Asn
20 25 30

(2) ANGABEN ZU SEQ ID NO: 13:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 47 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 53-99

(ix) MERKMAL:
(A) NAME/SCHLÜSSEL: Modified-site
(B) LAGE:10
(D) SONSTIGE ANGABEN:/product= "Xaa = Met(oxidiert)"

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 13:

Glu Asp Ala Ala Leu Thr Ile Tyr Glu Xaa Leu Gln Asn Ile Phe Ala
1 5 10 15

Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn Glu Thr Ile Val
20 25 30

Glu Asn Leu Leu Ala Asn Val Tyr His Gln Ile Asn His Leu Lys
35 40 45

(2) ANGABEN ZU SEQ ID NO: 14:

(i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 47 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: Einzelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Peptid

(viii) POSITION IM GENOM:
(B) KARTENPOSITION: 53-99

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 14:

Glu Asp Ala Ala Leu Thr Ile Tyr Glu Met Leu Gln Asn Ile Phe Ala
1 5 10 15

Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn Glu Thr Ile Val
20 25 30

Glu Asn Leu Leu Ala Asn Val Tyr His Gln Ile Asn His Leu Lys
35 40 45

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/21 A61K9/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | WO 98 28007 A (BIOGEN, INC.) 2 July 1998 | 1-15,18, 20,21, 23-26 |
| Y | see page 3, line 23 - line 32; claims 1-9,20,23,27-35,41-43; examples 2,4,6,7<br>see page 12, line 4 - line 8<br>see page 12, line 10 - page 13, line 7; table 1<br>see page 16, line 7 - line 12<br>--- | 16,17, 19,22 |
| Y | US 5 358 708 A (S.T. PATEL)<br>25 October 1994<br>cited in the application<br>see column 2, line 45 - line 57; example 1<br>see column 3, line 59 - line 66<br>---<br>-/-- | 16,17 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 11 February 1999 | 19/02/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Ryckebosch, A |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | EP 0 529 300 A (BIOFERON BIOCHEMISCHE SUBSTANZEN GMBH & CO) 3 March 1993<br>cited in the application<br>see claims 1,12,24-26,28; example 3<br>see page 6, line 6 - line 11 | 19,22 |
| A | EP 0 374 257 A (TORAY INDUSTRIES, INC.) 27 June 1990<br>see page 3, line 4 - line 18; claims 1-13<br>see page 5, line 15 - line 21<br>see page 14 | 1-26 |

Information on patent family members

International Application No
PCT/EP 98/06065

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9828007 A | 02-07-1998 | AU 5619198 A | 17-07-1998 |
| US 5358708 A | 25-10-1994 | NONE | |
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A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
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| Kategorie* | Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile | Betr. Anspruch Nr. |
|---|---|---|
| P,X | WO 98 28007 A (BIOGEN, INC.) 2. Juli 1998 | 1-15,18, 20,21, 23-26 |
| Y | siehe Seite 3, Zeile 23 - Zeile 32; Ansprüche 1-9,20,23,27-35,41-43; Beispiele 2,4,6,7<br>siehe Seite 12, Zeile 4 - Zeile 8<br>siehe Seite 12, Zeile 10 - Seite 13, Zeile 7; Tabelle 1<br>siehe Seite 16, Zeile 7 - Zeile 12 | 16,17, 19,22 |
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[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[X] Siehe Anhang Patentfamilie

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C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

| Kategorie* | Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile | Betr. Anspruch Nr. |
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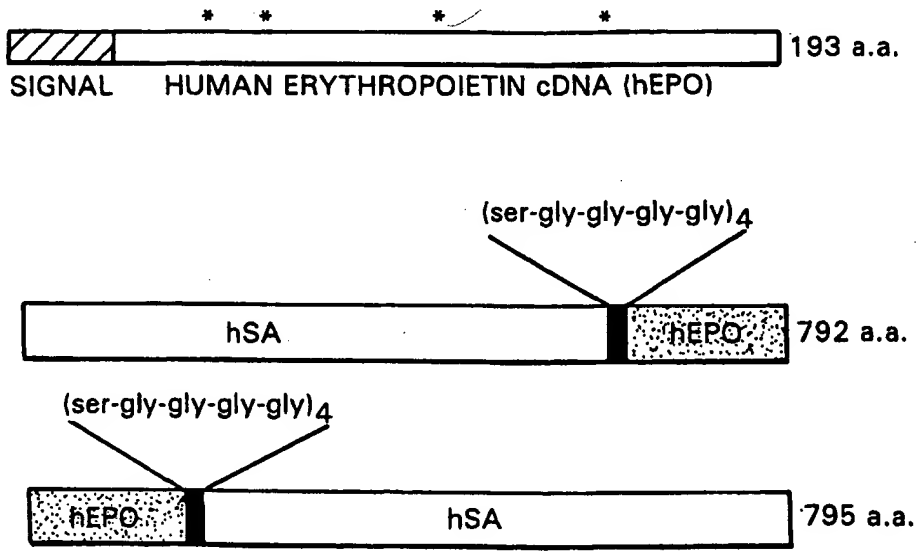


(57) Abstract

Erythropoietin analog-human serum albumin (EPOa-hSA) fusion protein and methods of making and using the fusion protein.

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# ERYTHROPOIETIN ANALOG-HUMAN SERUM ALBUMIN FUSION

## *Background of the Invention*

The invention relates to erythropoietin analog-human serum albumin (EPOa-hSA) fusion proteins, nucleic acids which encode EPOa-hSA fusion proteins, and methods of making and using EPOa-hSA fusion proteins and nucleic acids.

## *Summary of the Invention*

In general, the invention features, an EPOa-hSA fusion protein, wherein at least one amino acid residue of the EPOa moiety of the fusion protein is altered such that a site which serves as a site for glycosylation in erythropoietin (EPO) does not serve as a site for glycosylation in the EPOa, e.g., an EPOa-hSA fusion protein in which at least one amino acid residue which can serve as a glycosylation site in erythropoietin is altered, e.g., by substitution or deletion, such that it does not serve as a glycosylation site.

In a preferred embodiment, the EPOa-hSA fusion protein has the formula: R1-L-R2; R2-L-R1; or R1-L-R2-L-R1, wherein R1 is an EPOa amino acid sequence, L is a peptide linker and R2 is human serum albumin amino acid sequence. Preferably, R1 and R2 are covalently linked via the peptide linker.

In a preferred embodiment: an amino acid residue of EPO which serves as an attachment point for glycosylation has been deleted; an amino acid residue of EPO which serves as a site for glycosylation has been replaced with an amino acid residue which does not serve as a site for glycosylation; the amino acid residue which is altered is selected from the group consisting of amino acid residues Asn24, Asn38, Asn83 and Ser126; the glycosylation site at amino acid residue Ser126 and at least one additional N-linked glycosylation site selected from the group consisting of Asn24, Asn38 and Asn83 are altered; a glycosylation site which provides for N-linked glycosylation is altered by replacing an Asn residue with an amino acid residue other than it, e.g., Gln; a glycosylation site which provides for O-linked glycosylation is altered by replacing a Ser residue with an amino acid residue other than it, e.g., Ala.

In preferred embodiments, the EPOa-hSA fusion protein is made in a mammary gland of a transgenic mammal, e.g., a ruminant, e.g., a goat.

In preferred embodiments, the EPOa-hSA fusion protein is secreted into the milk of a transgenic mammal, e.g., a ruminant, e.g., a goat.

In preferred embodiments, the EPOa-hSA fusion protein is made, in a transgenic animal, under the control of a mammary gland specific promoter, e.g., a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can be a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter. Preferably, the promoter is a goat β casein promoter.

In preferred embodiments, the EPOa-hSA fusion protein, in a transgenic animal, and is secreted into the milk of a transgenic mammal at concentrations of at least about 0.2 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml or higher.

In a preferred embodiment, amino acid residue Asn24 has been altered, e.g., substituted or deleted. Preferably, the amino acid residue Asn24 has been replaced with Gln.

In a preferred embodiment, amino acid residue Asn38 has been altered, e.g., substituted or deleted. Preferably, amino acid residue Asn38 has been replaced with Gln.

In a preferred embodiment, amino acid residue Asn83 has been altered, e.g., substituted or deleted. Preferably, the amino acid residue Asn83 has been replaced with Gln.

In yet another embodiment, amino acid residue Ser126 has been altered, e.g., substituted or deleted. Preferably, the amino acid residue Ser126 has been replaced with Ala.

In a preferred embodiment: each of amino acid residue Asn24, Asn38, Asn83 and Ser126 has been altered, e.g., substituted or deleted, such that it does not serve as a glycosylation site; each of the amino acid residues Asn24, Asn28, Asn83 and Ser126 has, respectively, been replaced with Gln, Gln, Gln, and Ala.

In a preferred embodiment, the fusion protein includes a peptide linker and the peptide linker has one or more of the following characteristics: a) it allows for the rotation of the erythropoietin analog amino acid sequence and the human serum albumin amino acid sequence relative to each other; b) it is resistant to digestion by proteases; and c) it does not interact with the erythropoietin analog or the human serum albumin.

In a preferred embodiment: the fusion protein includes a peptide linker and the peptide linker is 5 to 60, more preferably, 10 to 30, amino acids in length; the peptide linker is 20 amino acids in length; the peptide linker is 17 amino acids in length; each of the amino acids in the peptide linker is selected from the group consisting of Gly, Ser, Asn, Thr and Ala; the peptide linker includes a Gly-Ser element.

In a preferred embodiment, the fusion protein includes a peptide linker and the peptide linker includes a sequence having the formula $(Ser\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Gly)_y$ wherein y is 1, 2, 3, 4, 5, 6, 7, or 8. Preferably, the peptide linker includes a sequence having the

formula (Ser-Gly-Gly-Gly-Gly)$_3$. Preferably, the peptide linker includes a sequence having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro).

In a preferred embodiment, the fusion protein includes a peptide linker and the peptide linker includes a sequence having the formula (Ser-Ser-Ser-Ser-Gly)$_y$ wherein y is 1, 2, 3, 4, 5, 6, 7, or 8. Preferably, the peptide linker includes a sequence having the formula ((Ser-Ser-Ser-Ser-Gly)$_3$-Ser-Pro).

In another aspect, the invention features, an EPOa-hSA fusion protein wherein the EPOa includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO (i.e., only amino acids 24, 38, 83, and 126 differ from wild type).

In another aspect, the invention features, an EPOa-hSA fusion protein which includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In another aspect, the invention features, an EPOa-hSA fusion protein which includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, an isolated nucleic acid having a nucleotide sequence which encodes an EPOa-hSA fusion protein described herein, e.g., an EPOa-hSA fusion protein wherein at least one amino acid residue is altered such that a site which serves as a site for glycosylation in EPO does not serve as a site for glycosylation in the EPOa, e.g., an EPOa-hSA fusion protein in which at least one amino acid residue of the encoded EPOa-hSA which can serve as a glycosylation site in

erythropoietin is altered, e.g., by substitution or deletion, such that it does not serve as a glycosylation site.

In another aspect, the invention features, a nucleic acid which encodes an EPOa-hSA fusion protein wherein the EPOa includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a nucleic acid which encodes an EPOa-hSA fusion protein which includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In another aspect, the invention features, a nucleic acid which encodes an EPOa-hSA fusion protein which includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, an expression vector or a construct which includes a nucleic acid of the invention.

In a preferred embodiment, the vector or construct further includes: a promoter; a selectable marker; an origin of replication; or a DNA homologous to a species other than human, e.g., goat DNA.

In preferred embodiments, the promoter is a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter is a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter. Preferably, the promoter is a goat β casein promoter.

In another aspect, the invention features, a cell which includes a vector or nucleic acid of the invention.

In another aspect, the invention features, a method of making an EPOa-hSA fusion in a nucleic acid construct or a vector. The method includes, forming in the construct or vector, a sequence in which a nucleic acid which encodes an erythropoietin analog is linked in frame to a nucleic acid which encodes human serum albumin.

In another aspect, the invention features, a method for making an EPOa-hSA fusion protein, e.g., from a cultured cell. The method includes supplying a cell which includes a nucleic acid which encodes an EPOa-hSA fusion protein, and expressing the EPOa-hSA fusion protein from the nucleic acid, thereby making the EPOa-hSA fusion protein.

In a preferred embodiment, the cell is a mammalian, yeast, plant, insect, or bacterial cell. Suitable mammalian cells include CHO cells or other similar expression systems.

In a preferred embodiment, the cell is a microbial cell, a cultured cell, or a cell from a cell line.

In a preferred embodiment, the EPOa-hSA fusion protein is released into culture medium.

In a preferred embodiment, the EPOa-hSA is released into culture medium and the method further includes purifying the EPOa-hSA fusion protein from culture medium.

In a preferred embodiment, the EPOa includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)3-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

The invention also includes a cultured cell which includes a nucleic acid which encodes an EPOa-hSA fusion protein, e.g., an EPOa-hSA fusion protein described herein. The invention also includes methods of making such cells, e.g., by introducing into the cell, or forming in the cell, a nucleic acid which encodes an EPOa-hSA fusion protein, e.g., an EPOa-hSA fusion protein described herein.

In another aspect, the invention features, a method of making an EPOa-hSA fusion protein, e.g., an EPOa-hSA described herein. The method includes providing a transgenic organism which includes a transgene which directs the expression of EPOa-hSA fusion protein; allowing the transgene to be expressed; and, preferably, recovering a transgenically produced EPOa-hSA fusion protein, e.g., from the organism or from a product produced by the organism.

In a preferred embodiment, the transgenic organism is a transgenic animal, e.g., a transgenic mammal, e.g., a transgenic dairy animal, e.g., a transgenic goat or a transgenic cow.

In a preferred embodiment, the EPOa-hSA fusion protein is secreted into a bodily fluid and the method further includes purifying the EPOa-hSA fusion protein from the bodily fluid.

In a preferred embodiment, the transgenically produced EPOa-hSA fusion protein is made in a mammary gland of a transgenic mammal, preferably under the control of a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can be a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter. Preferably, the promoter is a goat β casein promoter.

In preferred embodiments, the EPOa-hSA fusion protein is made in a mammary gland of the transgenic mammal, e.g., a ruminant, e.g., a dairy animal, e.g., a goat or cow.

In preferred embodiments, the EPOa-hSA fusion protein is secreted into the milk of a transgenic mammal at concentrations of at least about 0.2 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml or higher.

In preferred embodiments the method further includes recovering EPOa-hSA fusion protein from the organism or from a product produced by the organism, e.g., milk, seeds, hair, blood, eggs, or urine.

In yet another embodiment, the EPOa-hSA fusion protein is produced in a transgenic plant.

In a preferred embodiment, the erythropoietin analog includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a method of making a transgenic EPOa-hSA fusion protein, e.g., an EPOa-hSA fusion described herein. The method includes providing a transgenic animal, e.g., goat or a cow, which includes a transgene which provides for the expression of the EPOa-hSA fusion protein; allowing the transgene to be expressed; and, preferably, recovering EPOa-hSA fusion protein, from the milk of the transgenic animal.

In preferred embodiments, the EPOa-hSA fusion protein is made in a mammary gland of the transgenic mammal, e.g., a ruminant, e.g., a goat or a cow.

In preferred embodiments, the EPOa-hSA fusion protein is secreted into the milk of the transgenic mammal, e.g., a ruminant, e.g., a dairy animal, e.g., a goat or a cow.

In preferred embodiments, the EPOa-hSA fusion protein is made under the control of a mammary gland specific promoter, e.g., a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can be a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter. Preferably, the promoter is a goat β casein promoter.

In preferred embodiments, the EPOa-hSA fusion protein is secreted into the milk of a transgenic mammal at concentrations of at least about 0.2 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml or higher.

In a preferred embodiment, the EPOa includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a method for providing a transgenic preparation which includes an EPOa-hSA fusion protein, e.g., an EPOa-hSA fusion protein described herein, in the milk of a transgenic mammal. The method includes: providing a transgenic mammal having an EPOa-hSA fusion protein protein-coding sequence operatively linked to a promoter sequence that results in the expression of the protein-coding sequence in mammary gland epithelial cells, allowing the fusion protein to be expressed, and obtaining milk from the mammal, thereby providing the transgenic preparation.

In a preferred embodiment, the EPOa-hSA fusion protein-coding sequence operatively linked to a promoter sequence is introduced into the germline of the transgenic mammal.

In a preferred embodiment, the erythropoietin analog includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a

peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a method for providing a transgenic preparation which includes an EPOa-hSA fusion protein, e.g., an EPOa-hSA fusion protein described herein, in the milk of a transgenic goat or transgenic cow. The method includes providing a transgenic goat or cow having an EPOa-hSA fusion protein-coding sequence operatively linked to a promoter sequence that results in the expression of the protein-coding sequence in mammary gland epithelial cells, allowing the fusion protein to be expressed, and obtaining milk from the goat or cow, thereby providing the transgenic preparation.

In a preferred embodiment, the erythropoietin analog includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a transgenic organism, which includes a transgene which encodes an EPOa-hSA fusion protein, e.g., an EPOa-hSA fusion protein described herein.

In a preferred embodiment, the transgenic organism is a transgenic plant or animal. Preferred transgenic animals include: mammals; birds; reptiles; marsupials; and amphibians. Suitable mammals include: ruminants; ungulates; domesticated mammals; and dairy animals. Particularly preferred animals include: mice, goats, sheep, camels, rabbits, cows, pigs, horses, oxen, and llamas. Suitable birds include chickens, geese, and turkeys. Where the transgenic protein is secreted into the milk of a transgenic animal, the animal should be able to produce at least 1, and more preferably at least 10, or 100, liters of milk per year.

In preferred embodiments, the EPOa-hSA fusion protein is under the control of a mammary gland specific promoter, e.g., a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can be a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter. Preferably, the promoter is a goat β casein promoter.

In preferred embodiments, the EPOa-hSA fusion protein is secreted into the milk at concentrations of at least about 0.2 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml or higher.

In a preferred embodiment, the EPOa includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula

((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a transgenic cow, goat or sheep, which includes a transgene which encodes an EPOa-hSA fusion protein, e.g., an EPOa-hSA fusion protein described herein.

In preferred embodiments, the EPOa-hSA fusion protein is under the control of a mammary gland specific promoter, e.g., a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can be a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter. Preferably, the promoter is a goat β casein promoter.

In preferred embodiments, the EPOa-hSA fusion protein is secreted into the milk at concentrations of at least about 0.2 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml or higher.

In a preferred embodiment, the EPOa includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a herd of transgenic animals having at least one female and one male transgenic animal, wherein each animal includes an EPOa-hSA fusion protein transgene, e.g., a transgene which encodes an EPOa-hSA fusion protein described herein.

In a preferred embodiment, a transgenic animal of the herd is a mammal, bird, reptile, marsupial or amphibian. Suitable mammals include: ruminants; ungulates; domesticated mammals; and dairy animals. Particularly preferred animals include: mice, goats, sheep, camels, rabbits, cows, pigs, horses, oxen, and llamas. Suitable birds include chickens, geese, and turkeys. Where the transgenic protein is secreted into the milk of a transgenic animal, the animal should be able to produce at least 1, and more preferably at least 10, or 100, liters of milk per year.

In preferred embodiments, the EPOa-hSA fusion protein is under the control of a mammary gland specific promoter, e.g., a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can is a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter.

In preferred embodiments, the EPOa-hSA fusion protein is secreted into the milk at concentrations of at least about 0.2 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml or higher.

In a preferred embodiment, the EPOa includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a pharmaceutical composition having a therapeutically effective amount of an EPOa-hSA fusion protein, e.g., an EPOa-hSA fusion protein described herein, and a pharmaceutically acceptable carrier.

In a preferred embodiment, the composition includes milk.

In a preferred embodiment, the EPOa includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a kit having an EPOa-hSA fusion protein, e.g., an EPOa-hSA fusion protein described herein, packaged with instructions for treating a subject in need of erythropoietin.

In a preferred embodiment, the subject is a patient suffering from anemia associated with renal failure, chronic disease, HIV infection, blood loss or cancer.

In another preferred embodiment, the subject is a preoperative patient.

In a preferred embodiment, the erythropoietin analog includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a purified preparation of an EPOa-hSA fusion protein, e.g., an EPO-hSA fusion protein described herein.

In preferred embodiments, the preparation includes at least 1, 10, 100 or 1000 micrograms of EPOa-hSA fusion protein. In preferred embodiments, the preparation includes at least 1, 10, 100 or 1000 milligrams of EPOa-hSA fusion protein.

In another aspect, the invention features, an EPOa-hSA fusion protein, or a purified preparation thereof, wherein the erythropoietin analog includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In preferred embodiments, the preparation includes at least 1, 10, 100 or 1000 micrograms of EPOa-hSA fusion protein. In preferred embodiments, the preparation includes at least 1, 10, 100 or 1000 milligrams of EPOa-hSA fusion protein.

In another aspect, the invention features, an EPOa-hSA fusion protein, or a purified preparation thereof, which includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In preferred embodiments, the preparation includes at least 1, 10, 100 or 1000 micrograms of EPOa-hSA fusion protein. In preferred embodiments, the preparation includes at least 1, 10, 100 or 1000 milligrams of EPOa-hSA fusion protein.

In another aspect, the invention features, an EPOa-hSA fusion protein, or a purified preparation thereof, which includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In preferred embodiments, the preparation includes at least 1, 10, or 100 milligrams of EPOa-hSA fusion protein. In preferred embodiments, the preparation includes at least 1, 10, or 100 grams of EPOa-hSA fusion protein.

In another aspect, the invention features, a method of treating a subject, e.g., a human, in need of erythropoietin. The method includes administering a therapeutically effective amount of an EPOa-hSA fusion protein, e.g., an EPO-hSA fusion protein described herein, to the subject.

In a preferred embodiment, the subject is a patient suffering from anemia associated with renal failure, chronic disease, HIV infection, blood loss or cancer.

In another preferred embodiment, the subject is a preoperative patient.

In preferred embodiments the EPOa-hSA is administered repeatedly, e.g., at least two, three, five, or 10 times.

In a preferred embodiment, the erythropoietin analog includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a method of treating a subject in need of erythropoietin. The method includes delivering or providing a nucleic acid encoding an EPOa-hSA fusion protein, e.g., a fusion protein described herein, to the subject.

In a preferred embodiment, the nucleic acid is delivered to a target cell of the subject.

In a preferred embodiment, the nucleic acid is delivered or provided in a biologically effective carrier, e.g., an expression vector.

In a preferred embodiment, the nucleic acid is delivered or provided in a cell, e.g., an autologous, allogeneic, or xenogeneic cell.

In a preferred embodiment, the EPOa includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, a method of making a transgenic organism which has an EPOa-hSA transgene. The method includes providing or forming in a cell of an organism, an EPOa-hSA transgene, e.g., a transgene which encodes an EPOa-hSA fusion protein described herein; and allowing the cell, or a descendent of the cell, to give rise to a transgenic organism.

In a preferred embodiment, the transgenic organism is a transgenic plant or animal. Preferred transgenic animals include: mammals; birds; reptiles; marsupials; and amphibians. Suitable mammals include: ruminants; ungulates; domesticated mammals; and dairy animals. Particularly preferred animals include: mice, goats, sheep, camels, rabbits, cows, pigs, horses, oxen, and llamas. Suitable birds include chickens, geese, and turkeys. Where the transgenic protein is secreted into the milk of a transgenic animal, the animal should be able to produce at least 1, and more preferably at least 10, or 100, liters of milk per year.

In preferred embodiments, the EPOa-hSA fusion protein is under the control of a mammary gland specific promoter, e.g., a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can be a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter. Preferably, the promoter is a goat β casein promoter.

In preferred embodiments, the organism is a mammal, and the EPOa-hSA fusion protein is secreted into the milk of the transgenic animal at concentrations of at least about 0.2 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml or higher.

In a preferred embodiment, the EPOa includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In a preferred embodiment, the EPOa-hSA fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

In a preferred embodiment, the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, e.g., a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, an erythropoietin analog (EPOa) protein, or a purified preparation thereof, e.g., the EPOa moiety of an EPOa-hSA fusion

protein described herein, wherein at least one amino acid residue is altered such that a site which serves as a site for glycosylation in EPO, does not serve as a site for glycosylation in the EPOa, e.g., an EPOa in which at least one amino acid residue which can serve as a glycosylation site in erythropoietin is altered, e.g., by substitution or deletion, such that it does not serve as a glycosylation site.

In a preferred embodiment, the erythropoietin analog includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

In a preferred embodiment the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

In another aspect, the invention features, an isolated nucleic acid having a nucleotide sequence which encodes an EPOa described herein.

In another aspect, the invention features, an expression vector or a construct which includes an EPOa nucleic acid described herein.

In a preferred embodiment, the vector or construct further includes: a promoter; a selectable marker; an origin of replication; or a DNA homologous to a species other than human, e.g., goat DNA.

In another aspect, the invention features, a cell which includes a vector or construct which includes an EPOa nucleic acid described herein.

A purified preparation, substantially pure preparation of a polypeptide, or an isolated polypeptide as used herein, means a polypeptide that has been separated from at least one other protein, lipid, or nucleic acid with which it occurs in the cell or organism which expresses it, e.g., from a protein, lipid, or nucleic acid in a transgenic animal or in a fluid, e.g., milk, or other substance, e.g., an egg, produced by a transgenic animal. The polypeptide is preferably separated from substances, e.g., antibodies or gel matrix, e.g., polyacrylamide, which are used to purify it. The polypeptide preferably constitutes at least 10, 20, 50 70, 80 or 95% dry weight of the purified preparation. Preferably, the preparation contains: sufficient polypeptide to allow protein sequencing; at least 1, 10, or 100 μg of the polypeptide; at least 1, 10, or 100 mg of the polypeptide.

As used herein, "human serum albumin" or "hSA" refers to a polypeptide having the amino acid sequence described in Minghetti et al. *J. Biol. Chem.* 261:6747-6757, 1986; Lawn et al. *Nucl. Acids Res.* 9:6103, 1981. In preferred embodiments, sequence variations are included wherein one or up to two, five, 10, or 20 amino acid residues have been substituted, inserted or deleted. Variants will have substantially the same

immunogenicity, in, e.g., mice, rats, rabbits, primates, baboons, or humans, as does hSA. Variants, when incorporated into a fusion protein which includes EPOa, will result in an EPOa-hSA a fusion which has similar clearance time, in e.g., mice, rabbits, or humans, and activity as does a fusion protein which includes the EPOa and hSA.

As used herein, "erythropoietin" or "EPO" refers to a glycoprotein hormone involved in the maturation of erythroid progenitor cells into erythrocytes. The sequence of EPO can be found in Powell, J.S., et al., *Proc. Natl. Acad. Sci. USA*, 83:6465-6469 (1986).

A substantially pure nucleic acid, is a nucleic acid which is one or both of: not immediately contiguous with either one or both of the sequences, e.g., coding sequences, with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the nucleic acid is derived; or which is substantially free of a nucleic acid sequence with which it occurs in the organism from which the nucleic acid is derived. The term includes, for example, a recombinant DNA which is incorporated into a vector, e.g., into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other DNA sequences. Substantially pure DNA also includes a recombinant DNA which is part of a hybrid gene encoding additional EPOa-hSA fusion protein sequence.

Homology, or sequence identity, as used herein, refers to the sequence similarity between two polypeptide molecules or between two nucleic acid molecules. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are homologous at that position (i.e., as used herein amino acid or nucleic acid "homology" is equivalent to amino acid or nucleic acid "identity"). The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = # of identical positions/total # of positions x 100). For example, if 6 of 10, of the positions in two sequences are matched or homologous then the two sequences are 60% homologous or have 60% sequence identity. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology or sequence identity. Generally, a comparison is made when two sequences are aligned to give maximum homology or sequence identity.

The comparison of sequences and determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci.* USA 87:2264-68, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90:5873-77. Such

an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to ITALY nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to ITALY protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

The terms peptides, proteins, and polypeptides are used interchangeably herein.

As used herein, the term transgene means a nucleic acid sequence (encoding, e.g., one or more EPOa-hSA fusion protein polypeptides), which is introduced into the genome of a transgenic organism. A transgene can include one or more transcriptional regulatory sequences and other nucleic acid, such as introns, that may be necessary for optimal expression and secretion of a nucleic acid encoding the fusion protein. A transgene can include an enhancer sequence. An EPOa-hSA fusion protein sequence can be operatively linked to a tissue specific promoter, e.g., mammary gland specific promoter sequence that results in the secretion of the protein in the milk of a transgenic mammal, a urine specific promoter, or an egg specific promoter.

As used herein, the term "transgenic cell" refers to a cell containing a transgene.

A transgenic organism, as used herein, refers to a transgenic animal or plant.

As used herein, a "transgenic animal" is a non-human animal in which one or more, and preferably essentially all, of the cells of the animal contain a transgene introduced by way of human intervention, such as by transgenic techniques known in the art. The transgene can be introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus.

As used herein, a "transgenic plant" is a plant, preferably a multi-celled or higher plant, in which one or more, and preferably essentially all, of the cells of the plant

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Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

***Detailed Description***

The drawings are first described.

*Figure 1* is a schematic diagram of EPOa-hSA fusion constructs. Asterisks indicate sites of glycosylation of native human erythropoietin.

*Figure 2* is a photograph depicting the Western blot analysis of *COS7* cells transiently transfected with EPOa-hSA cDNA constructs.

Glycosylation

EPO is a glycoprotein hormone which mediates the maturation of erythroid progenitor cells into erythrocytes. It plays an important role in regulating the level of red blood cells in circulation. Naturally occurring EPO is produced by the liver during fetal life and by the kidney in adults and circulates in the blood and stimulates the production of red blood cells in the bone marrow.

Many cell surface and secretory proteins produced by eucaryotic cells are modified by the attachment of one or more oligosaccharide groups. The modification, referred to as glycosylation, can dramatically affect the physical properties of proteins and can be important in protein stability, secretion, and localization. Glycosylation occurs at specific locations along the polypeptide backbone. There are usually two major types of glycosylation: glycosylation characterized by O-linked oligosaccharides, which are attached to serine or threonine residues; and glycosylation characterized by N-linked oligosaccharides, which are attached to asparagine residues in an Asn-X-Ser/Thr sequence, where X can be any amino acid except proline. N-acetylneuramic acid (hereafter referred to as sialic acid) is usually the terminal residue of both N-linked and O-linked oligosaccharides.

Human urinary derived EPO contains three N-linked and one O-linked oligosaccharide chains. N-linked glycosylation occurs at asparagine residues located at positions 24, 38 and 83 while O-linked glycosylation occurs at a serine residue located at position 126 (Lai et al. *J. Biol. Chem.* 261, 3116 (1986); Broudy et al, *Arch. Biochem. Biophys.* 265, 329 (1988).

As described herein, EPO analogs of the invention have been modified so that glycosylation at one, two, three, or all of these sites is abolished, e.g., by substitution or deletion of an amino acid residue.

EPO Glycosylation Analogs

An EPO analog can differ from a naturally occurring or recombinant EPO at one or more of the following amino acids: Asn24, Asn38, Asn83 or Ser126. In an EPOa, the primary sequence can be altered such that one or more of these residues fails to support glycosylation.

Preferred analogs are listed below, wherein, Xaa is an amino acid which does not support attachment of a sugar residue, e.g., Gln or Ala

| | 24 | 38 | 83 | 126 |
|---|---|---|---|---|
| wild-type | Asn | Asn | Asn | Ser |
| EPOa-1 | Xaa | Xaa | Xaa | Xaa |
| EPOa-2 | Asn | Xaa | Xaa | Xaa |
| EPOa-3 | Xaa | Asn | Xaa | Xaa |
| EPOa-4 | Xaa | Xaa | Asn | Xaa |
| EPOa-5 | Xaa | Xaa | Xaa | Ser |
| EPOa-6 | Asn | Asn | Xaa | Xaa |
| EPOa-7 | Asn | Xaa | Asn | Ser |
| EPOa-8 | Xaa | Asn | Asn | Xaa |
| EPOa-9 | Xaa | Asn | Asn | Ser |
| EPOa-10 | Xaa | Xaa | Asn | Ser |
| EPOa-11 | Xaa | Asn | Xaa | Ser |
| EPOa-12 | Asn | Xaa | Asn | Xaa |
| EPOa-13 | Asn | Xaa | Asn | Ser |
| EPOa-14 | Asn | Asn | Asn | Xaa |
| EPOa-15 | Asn | Asn | Xaa | Ser |

An EPOa can differ from EPO only at one or more or all of sites 24, 38, 83 and 126 or can have additional amino acid substitutions and/or deletions as discussed below.

EPOa-hSA Fusion Protein Coding Sequences

The preferred EPOa-hSA fusion has one EPOa linked to one hSA molecule but other conformations are within the invention. E.g., EPOa-hSA fusion proteins can have any of the following formula: $R_1$-L-$R_2$; $R_2$-L-$R_1$; $R_1$-L-$R_2$-L-$R_1$; or $R_2$-L-$R_1$-L-$R_2$; $R_1$-$R_2$; $R_2$-$R_1$; $R_1$-$R_2$-$R_1$; or $R_2$-$R_1$-$R_2$;wherein $R_1$ is an EPO analog, $R_2$ is hSA, and L is a peptide linker sequence.

EPOa and hSA domains are linked to each other, preferably via a linker sequence. The linker sequence should separate EPOa and hSA domains by a distance sufficient to ensure that each domain properly folds into its secondary and tertiary structures. Preferred linker sequences (1) should adopt a flexible extended conformation, (2) should not exhibit a propensity for developing an ordered secondary structure which could interact with the functional EPOa and hSA domains, and (3) should have minimal hydrophobic or charged character, which could promote interaction with the functional protein domains. Typical surface amino acids in flexible protein regions include Gly, Asn and Ser. Permutations of amino acid sequences containing Gly, Asn and Ser would be expected to satisfy the above criteria for a linker sequence. Other near neutral amino acids, such as Thr and Ala, can also be used in the linker sequence.

A linker sequence length of 20 amino acids can be used to provide a suitable separation of functional protein domains, although longer or shorter linker sequences may also be used. The length of the linker sequence separating EPOa and hSA can be from 5 to 500 amino acids in length, or more preferably from 5 to 100 amino acids in length. Preferably, the linker sequence is from about 5-30 amino acids in length. In preferred embodiments, the linker sequence is from about 5 to about 20 amino acids, and is advantageously from about 10 to about 20 amino acids. Amino acid sequences useful as linkers of EPOa and hSA include, but are not limited to, $(SerGly_4)_y$ wherein y is greater than or equal to 8, or $Gly_4SerGly_5Ser$. A preferred linker sequence has the formula $(SerGly_4)_4$. Another preferred linker has the sequence ((Ser-Ser-Ser-Ser-Gly)$_3$-Ser-Pro).

The EPOa and hSA proteins can be directly fused without a linker sequence. Linker sequences are unnecessary where the proteins being fused have non-essential N- or C-terminal amino acid regions which can be used to separate the functional domains and prevent steric interference. In preferred embodiments, the C-terminus of EPOa can be directly fused to the N-terminus of hSA or the C-terminus of hSA can be directly fused to the N-terminus of EPOa.

Recombinant Production

An EPOa-hSA fusion protein can be prepared with standard recombinant DNA techniques using a nucleic acid molecule encoding the fusion protein. A nucleotide sequence encoding a fusion protein can be synthesized by standard DNA synthesis methods.

A nucleic acid encoding a fusion protein can be introduced into a host cell, e.g., a cell of a primary or immortalized cell line. The recombinant cells can be used

to produce the fusion protein. A nucleic acid encoding a fusion protein can be introduced into a host cell, e.g., by homologous recombination. In most cases, a nucleic acid encoding the EPOa-hSA fusion protein is incorporated into a recombinant expression vector.

The nucleotide sequence encoding a fusion protein can be operatively linked to one or more regulatory sequences, selected on the basis of the host cells to be used for expression. The term "operably linked" means that the sequences encoding the fusion protein compound are linked to the regulatory sequence(s) in a manner that allows for expression of the fusion protein. The term "regulatory sequence" refers to promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990), the content of which are incorporated herein by reference. Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cells, those that direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences) and those that direct expression in a regulatable manner (*e.g.*, only in the presence of an inducing agent). It will be appreciated by those skilled in the art that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed, the level of expression of fusion protein desired, and the like. The fusion protein expression vectors can be introduced into host cells to thereby produce fusion proteins encoded by nucleic acids.

Recombinant expression vectors can be designed for expression of fusion proteins in prokaryotic or eukaryotic cells. For example, fusion proteins can be expressed in bacterial cells such as *E. coli*, insect cells (e.g., in the baculovirus expression system), yeast cells or mammalian cells. Some suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA). Baculovirus vectors available for expression of fusion proteins in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.*, (1983) *Mol. Cell. Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) *Virology* 170:31-39).

Examples of mammalian expression vectors include pCDM8 (Seed, B., (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often

provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40.

In addition to the regulatory control sequences discussed above, the recombinant expression vector can contain additional nucleotide sequences. For example, the recombinant expression vector may encode a selectable marker gene to identify host cells that have incorporated the vector. Moreover, to facilitate secretion of the fusion protein from a host cell, in particular mammalian host cells, the recombinant expression vector can encode a signal sequence operatively linked to sequences encoding the amino-terminus of the fusion protein such that upon expression, the fusion protein is synthesized with the signal sequence fused to its amino terminus. This signal sequence directs the fusion protein into the secretory pathway of the cell and is then cleaved, allowing for release of the mature fusion protein (*i.e.*, the fusion protein without the signal sequence) from the host cell. Use of a signal sequence to facilitate secretion of proteins or peptides from mammalian host cells is known in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells *via* conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection and viral-mediated transfection. Suitable methods for transforming or transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

Often only a small fraction of mammalian cells integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) can be introduced into the host cells along with the gene encoding the fusion protein. Preferred selectable markers include those that confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding the fusion protein or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Transgenic Mammals

Methods for generating non-human transgenic animals are described herein. DNA constructs can be introduced into the germ line of a mammal to make a transgenic mammal. For example, one or several copies of the construct can be incorporated into the genome of a mammalian embryo by standard transgenic techniques.

It is often desirable to express the transgenic protein in the milk of a transgenic mammal. Mammals that produce large volumes of milk and have long lactating periods are preferred. Preferred mammals are ruminants, e.g., cows, sheep, camels or goats, e.g., goats of Swiss origin, e.g., the Alpine, Saanen and Toggenburg breed goats. Other preferred animals include oxen, rabbits and pigs.

In an exemplary embodiment, a transgenic non-human animal is produced by introducing a transgene into the germline of the non-human animal. Transgenes can be introduced into embryonal target cells at various developmental stages. Different methods are used depending on the stage of development of the embryonal target cell. The specific line(s) of any animal used should, if possible, be selected for general good health, good embryo yields, good pronuclear visibility in the embryo, and good reproductive fitness.

Introduction of the EPOa-hSA fusion protein transgene into the embryo can be accomplished by any of a variety of means known in the art such as microinjection, electroporation, or lipofection. For example, an EPOa-hSA fusion protein transgene can be introduced into a mammal by microinjection of the construct into the pronuclei of the fertilized mammalian egg(s) to cause one or more copies of the construct to be retained in the cells of the developing mammal(s). Following introduction of the transgene construct into the fertilized egg, the egg can be incubated *in vitro* for varying amounts of time, or reimplanted into the surrogate host, or both. One common method is to incubate the embryos *in vitro* for about 1-7 days, depending on the species, and then reimplant them into the surrogate host.

The progeny of the transgenically manipulated embryos can be tested for the presence of the construct by Southern blot analysis of a segment of tissue. An embryo having one or more copies of the exogenous cloned construct stably integrated into the genome can be used to establish a permanent transgenic mammal line carrying the transgenically added construct.

Litters of transgenically altered mammals can be assayed after birth for the incorporation of the construct into the genome of the offspring. This can be done by hybridizing a probe corresponding to the DNA sequence coding for the fusion protein or a segment thereof onto chromosomal material from the progeny. Those mammalian progeny found to contain at least one copy of the construct in their genome are grown to

maturity. The female species of these progeny will produce the desired protein in or along with their milk. The transgenic mammals can be bred to produce other transgenic progeny useful in producing the desired proteins in their milk.

Transgenic females may be tested for protein secretion into milk, using an art-known assay technique, e.g., a Western blot or enzymatic assay.

Production of Transgenic Protein in the Milk of a Transgenic Animal

Milk Specific Promoters

Useful transcriptional promoters are those promoters that are preferentially activated in mammary epithelial cells, including promoters that control the genes encoding milk proteins such as caseins, beta lactoglobulin (Clark et al., (1989) *Bio/Technology 7:* 487-492), whey acid protein (Gorton et al. (1987) *Bio/Technology 5*: 1183-1187), and lactalbumin (Soulier et al., (1992) *FEBS Letts. 297:* 13). The alpha, beta, gamma or kappa casein gene promoter of any mammalian species can be used to provide mammary expression; a preferred promoter is the goat beta casein gene promoter (DiTullio, (1992) *Bio/Technology* 10:74-77). Milk-specific protein promoter or the promoters that are specifically activated in mammary tissue can be isolated from cDNA or genomic sequences. Preferably, they are genomic in origin.

DNA sequence information is available for mammary gland specific genes listed above, in at least one, and often in several organisms. See, e.g., Richards et al., *J. Biol. Chem.* 256, 526-532 (1981) (α-lactalbumin rat); Campbell et al., *Nucleic Acids Res.* 12, 8685-8697 (1984) (rat WAP); Jones et al., *J. Biol. Chem.* 260, 7042-7050 (1985) (rat β-casein); Yu-Lee & Rosen, *J. Biol. Chem.* 258, 10794-10804 (1983) (rat γ-casein); Hall, *Biochem. J.* 242, 735-742 (1987) (α-lactalbumin human); Stewart, *Nucleic Acids Res.* 12, 389 (1984) (bovine αs1 and κ casein cDNAs); Gorodetsky et al., *Gene* 66, 87-96 (1988) (bovine β casein); Alexander et al., *Eur. J. Biochem.* 178, 395-401 (1988) (bovine κ casein); Brignon et al., *FEBS Lett.* 188, 48-55 (1977) (bovine αS2 casein); Jamieson et al., *Gene* 61, 85-90 (1987), Ivanov et al., *Biol. Chem.* Hoppe-Seyler 369, 425-429 (1988), Alexander et al., *Nucleic Acids Res.* 17, 6739 (1989) (bovine β lactoglobulin); Vilotte et al., *Biochimie* 69, 609-620 (1987) (bovine α-lactalbumin). The structure and function of the various milk protein genes are reviewed by Mercier & Vilotte, *J. Dairy Sci.* 76, 3079-3098 (1993) (incorporated by reference in its entirety for all purposes). If additional flanking sequence are useful in optimizing expression, such sequences can be cloned using the existing sequences as probes. Mammary-gland specific regulatory sequences from different organisms can be obtained by screening

libraries from such organisms using known cognate nucleotide sequences, or antibodies to cognate proteins as probes.

Signal Sequences

Useful signal sequences are milk-specific signal sequences or other signal sequences which result in the secretion of eukaryotic or prokaryotic proteins. Preferably, the signal sequence is selected from milk-specific signal sequences, i.e., it is from a gene which encodes a product secreted into milk. Most preferably, the milk-specific signal sequence is related to the milk-specific promoter used in the expression system of this invention. The size of the signal sequence is not critical for this invention. All that is required is that the sequence be of a sufficient size to effect secretion of the desired recombinant protein, e.g., in the mammary tissue. For example, signal sequences from genes coding for caseins, e.g., alpha, beta, gamma or kappa caseins, beta lactoglobulin, whey acid protein, and lactalbumin are useful in the present invention. A preferred signal sequence is the goat β-casein signal sequence.

Signal sequences from other secreted proteins, e.g., proteins secreted by liver cells, kidney cell, or pancreatic cells can also be used.

DNA Constructs

An EPOa-hSA fusion protein can be expressed from a construct which includes a promoter specific for mammary epithelial cells, e.g., a casein promoter, e.g., a goat beta casein promoter, a milk-specific signal sequence, e.g., a casein signal sequence, e.g., a β-casein signal sequence, and a DNA encoding an EPOa-hSA fusion protein.

A construct can also include a 3' untranslated region downstream of the DNA sequence coding for the non-secreted protein. Such regions can stabilize the RNA transcript of the expression system and thus increases the yield of desired protein from the expression system. Among the 3' untranslated regions useful in the constructs of this invention are sequences that provide a poly A signal. Such sequences may be derived, e.g., from the SV40 small t antigen, the casein 3' untranslated region or other 3' untranslated sequences well known in the art. Preferably, the 3' untranslated region is derived from a milk specific protein. The length of the 3' untranslated region is not critical but the stabilizing effect of its poly A transcript appears important in stabilizing the RNA of the expression sequence.

A construct can include a 5' untranslated region between the promoter and the DNA sequence encoding the signal sequence. Such untranslated regions can be from the same control region from which promoter is taken or can be from a different gene, e.g.,

they may be derived from other synthetic, semi-synthetic or natural sources. Again their specific length is not critical, however, they appear to be useful in improving the level of expression.

A construct can also include about 10%, 20%, 30%, or more of the N-terminal coding region of a gene preferentially expressed in mammary epithelial cells. For example, the N-terminal coding region can correspond to the promoter used, e.g., a goat β-casein N-terminal coding region.

Prior art methods can include making a construct and testing it for the ability to produce a product in cultured cells prior to placing the construct in a transgenic animal. Surprisingly, the inventors have found that such a protocol may not be of predictive value in determining if a normally non-secreted protein can be secreted, e.g., in the milk of a transgenic animal. Therefore, it may be desirable to test constructs directly in transgenic animals, e.g., transgenic mice, as some constructs which fail to be secreted in CHO cells are secreted into the milk of transgenic animals.

Purification from milk

The transgenic protein can be produced in milk at relatively high concentrations and in large volumes, providing continuous high level output of normally processed peptide that is easily harvested from a renewable resource. There are several different methods known in the art for isolation of proteins from milk.

Milk proteins usually are isolated by a combination of processes. Raw milk first is fractionated to remove fats, for example, by skimming, centrifugation, sedimentation (H.E. Swaisgood, Developments in Dairy Chemistry, I: Chemistry of Milk Protein, Applied Science Publishers, NY, 1982), acid precipitation (U.S. Patent No. 4,644,056) or enzymatic coagulation with rennin or chymotrypsin (Swaisgood, *ibid.*). Next, the major milk proteins may be fractionated into either a clear solution or a bulk precipitate from which the specific protein of interest may be readily purified.

USSN 08/648,235 discloses a method for isolating a soluble milk component, such as a peptide, in its biologically active form from whole milk or a milk fraction by tangential flow filtration. Unlike previous isolation methods, this eliminates the need for a first fractionation of whole milk to remove fat and casein micelles, thereby simplifying the process and avoiding losses of recovery and bioactivity. This method may be used in combination with additional purification steps to further remove contaminants and purify the component of interest.

Production of Transgenic Protein in the Eggs of a Transgenic Animal

An EPOa-hSA fusion protein can be produced in tissues, secretions, or other products, e.g., an egg, of a transgenic animal. EPOa-hSA can be produced in the eggs of a transgenic animal, preferably a transgenic turkey, duck, goose, ostrich, guinea fowl, peacock, partridge, pheasant, pigeon, and more preferably a transgenic chicken, using methods known in the art (Sang et al., Trends Biotechnology, 12:415-20, 1994). Genes encoding proteins specifically expressed in the egg, such as yolk-protein genes and albumin-protein genes, can be modified to direct expression of EPOa-hSA.

Egg Specific Promoters

Useful transcriptional promoters are those promoters that are preferentially activated in the egg, including promoters that control the genes encoding egg proteins, e.g., ovalbumin, lysozyme and avidin. Promoters from the chicken ovalbumin, lysozyme or avidin genes are preferred. Egg-specific protein promoters or the promoters that are specifically activated in egg tissue can be from cDNA or genomic sequences. Preferably, the egg-specific promoters are genomic in origin.

DNA sequences of egg specific genes are known in the art (see, e.g., Burley et al., "The Avian Egg", John Wiley and Sons, p. 472, 1989, the contents of which are incorporated herein by reference). If additional flanking sequence are useful in optimizing expression, such sequences can be cloned using the existing sequences as probes. Egg specific regulatory sequences from different organisms can be obtained by screening libraries from such organisms using known cognate nucleotide sequences, or antibodies to cognate proteins as probes.

Transgenic Plants

An EPOa-hSA fusion protein can be expressed in a transgenic organism, e.g., a transgenic plant, e.g., a transgenic plant in which the DNA transgene is inserted into the nuclear or plastidic genome. Plant transformation is known as the art. See, in general, *Methods in Enzymology* Vol. 153 ("Recombinant DNA Part D") 1987, Wu and Grossman Eds., Academic Press and European Patent Application EP 693554.

Foreign nucleic acid can be introduced into plant cells or protoplasts by several methods. For example, nucleic acid can be mechanically transferred by microinjection directly into plant cells by use of micropipettes. Foreign nucleic acid can also be transferred into a plant cell by using polyethylene glycol which forms a precipitation complex with the genetic material that is taken up by the cell (Paszkowski et al. (1984) *EMBO J.* 3:2712-22). Foreign nucleic acid can be introduced into a plant cell by electroporation (Fromm et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:5824). In this technique, plant protoplasts are electroporated in the presence of plasmids or nucleic

acids containing the relevant genetic construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form a plant callus. Selection of the transformed plant cells with the transformed gene can be accomplished using phenotypic markers.

Cauliflower mosaic virus (CaMV) can be used as a vector for introducing foreign nucleic acid into plant cells (Hohn et al. (1982) "Molecular Biology of Plant Tumors," Academic Press, New York, pp. 549-560; Howell, U.S. Pat. No. 4,407,956). CaMV viral DNA genome is inserted into a parent bacterial plasmid creating a recombinant DNA molecule which can be propagated in bacteria. The recombinant plasmid can be further modified by introduction of the desired DNA sequence. The modified viral portion of the recombinant plasmid is then excised from the parent bacterial plasmid, and used to inoculate the plant cells or plants.

High velocity ballistic penetration by small particles can be used to introduce foreign nucleic acid into plant cells. Nucleic acid is disposed within the matrix of small beads or particles, or on the surface (Klein et al. (1987) *Nature* 327:70-73). Although typically only a single introduction of a new nucleic acid segment is required, this method also provides for multiple introductions.

A nucleic acid can be introduced into a plant cell by infection of a plant cell, an explant, a meristem or a seed with *Agrobacterium tumefaciens* transformed with the nucleic acid. Under appropriate conditions, the transformed plant cells are grown to form shoots, roots, and develop further into plants. The nucleic acids can be introduced into plant cells, for example, by means of the Ti plasmid of *Agrobacterium tumefaciens*. The Ti plasmid is transmitted to plant cells upon infection by *Agrobacterium tumefaciens*, and is stably integrated into the plant genome (Horsch et al. (1984) "Inheritance of Functional Foreign Genes in Plants," *Science* 233:496-498; Fraley et al. (1983) *Proc. Natl. Acad. Sci. USA* 80:4803).

Plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed so that whole plants are recovered which contain the transferred foreign gene. Some suitable plants include, for example, species from the genera Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Ciohorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hererocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaalia, Glycine, Lolium, Zea, Triticum, Sorghum, and Datura.

Plant regeneration from cultured protoplasts is described in Evans et al., "Protoplasts Isolation and Culture," *Handbook of Plant Cell Cultures* 1:124-176 (MacMillan Publishing Co. New York 1983); M.R. Davey, "Recent Developments in the Culture and Regeneration of Plant Protoplasts," *Protoplasts* (1983)-Lecture Proceedings, pp. 12-29, (Birkhauser, Basal 1983); P.J. Dale, "Protoplast Culture and Plant Regeneration of Cereals and Other Recalcitrant Crops," *Protoplasts* (1983)-Lecture Proceedings, pp. 31-41, (Birkhauser, Basel 1983); and H. Binding, "Regeneration of Plants," *Plant Protoplasts*, pp. 21-73, (CRC Press, Boca Raton 1985).

Regeneration from protoplasts varies from species to species of plants, but generally a suspension of transformed protoplasts containing copies of the exogenous sequence is first generated. In certain species, embryo formation can then be induced from the protoplast suspension, to the stage of ripening and germination as natural embryos. The culture media can contain various amino acids and hormones, such as auxin and cytokinins. It can also be advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Shoots and roots normally develop simultaneously. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

In vegetatively propagated crops, the mature transgenic plants can be propagated by the taking of cuttings or by tissue culture techniques to produce multiple identical plants for trialling, such as testing for production characteristics. Selection of a desirable transgenic plant is made and new varieties are obtained thereby, and propagated vegetatively for commercial sale. In seed propagated crops, the mature transgenic plants can be self crossed to produce a homozygous inbred plant. The inbred plant produces seed containing the gene for the newly introduced foreign gene activity level. These seeds can be grown to produce plants that have the selected phenotype. The inbreds according to this invention can be used to develop new hybrids. In this method a selected inbred line is crossed with another inbred line to produce the hybrid.

Parts obtained from a transgenic plant, such as flowers, seeds, leaves, branches, fruit, and the like are covered by the invention, provided that these parts include cells which have been so transformed. Progeny and variants, and mutants of the regenerated plants are also included within the scope of this invention, provided that these parts comprise the introduced DNA sequences. Progeny and variants, and mutants of the regenerated plants are also included within the scope of this invention.

Selection of transgenic plants or plant cells can be based upon a visual assay, such as observing color changes (e.g., a white flower, variable pigment production, and uniform color pattern on flowers or irregular patterns), but can also involve biochemical

assays of either enzyme activity or product quantitation. Transgenic plants or plant cells are grown into plants bearing the plant part of interest and the gene activities are monitored, such as by visual appearance (for flavonoid genes) or biochemical assays (Northern blots); Western blots; enzyme assays and flavonoid compound assays, including spectroscopy, see, Harborne et al. (Eds.), (1975) *The Flavonoids*, Vols. 1 and 2, [Acad. Press]). Appropriate plants are selected and further evaluated. Methods for generation of genetically engineered plants are further described in US Patent No. 5,283,184, US Patent No. 5, 482,852, and European Patent Application EP 693 554, all of which are hereby incorporated by reference.

Other Erythropoietin Analogs

Preferably, EPO analogs have one or more changes in the following amino acids: Asn24, Asn38, Asn83 or Ser126. EPO analogs can also have additional amino acid changes, as is discussed below.

In a preferred embodiment, the EPOa differs in amino acid sequence at up to 1, 2, 3, 5, or 10 residues, from the sequence of naturally occurring EPO protein. These changes can be in addition to changes at Asn24, Asn38, Asn83, and Ser126. In other preferred embodiments, the EPOa differs in amino acid sequence at up to 1, 2, 3, 5, or 10 % of the residues from a sequence of naturally occurring EPO protein. These changes can be in addition to changes at Asn24, Asn38, Asn 83, and Ser126. In preferred embodiments, the differences are such that the erythropoietin analog exhibits an erythropoietin biological activity when fused to hSA. In preferred embodiments, one or more, or all of the differences are conservative amino acid changes. In other preferred embodiments, one or more, or all of the differences are other than conservative amino acid changes.

In preferred embodiments, the EPOa is a fragment, e.g., a terminal fragment on a sequence from which an interval subsequence has been deleted, of a full length erythropoietin.

In preferred embodiments: the fragment is at least 50, 60, 80, 100 or 150 amino acids in length; the fragment has a biological activity of a naturally occurring erythropoietin; the fragment is either, an agonist or an antagonist, of a biological activity of a naturally occurring erythropoietin; the fragment can inhibit, e.g., competitively or non competitively inhibit, the binding of erythropoietin to a receptor.

In preferred embodiments, the fragment it has at least 60, and more preferably at least 70, 80, 90, 95, 99, or 100 % sequence identity with the corresponding amino acid sequence of naturally occurring erythropoietin.

In preferred embodiments, the fragment is a fragment of a vertebrate, e.g., a mammalian, e.g. a primate, e.g., a human erythropoietin.

In a preferred embodiment, the fragment differs in amino acid sequence at up to 1, 2, 3, 5, or 10 residues, from the corresponding residues of naturally occurring erythropoietin. These changes can be in addition to changes at Asn24, Asn38, Asn83, and Ser126. In other preferred embodiments, the fragment differs in amino acid sequence at up to 1, 2, 3, 5, or 10 % of the residues from the corresponding residues of naturally occurring erythropoietin. These changes can be in addition to changes at Asn24, Asn38, Asn83, and Ser126. In preferred embodiments, the differences are such that the fragment exhibits an erythropoietin biological activity when fused to hSA. In preferred embodiments, one or more, or all of the differences are conservative amino acid changes. In other preferred embodiments one or more, or all of the differences are other than conservative amino acid changes.

Polypeptides of the invention include those which arise as a result of alternative translational and postranslational events.

Numerous analogs of EPO are known in the art. The primary structure and activity of these variants can serve as guidance for the introduction of additional changes (in addition to changes which modify glycosylation) into an EPOa. Changes which reduce activity, or create glycosylation sites, should be avoided.

Some of the EPO analogs known in the art are outlined in Table 1 below.

TABLE 1

| EPO mutation | Loc. | Type | Effect | Source | Reference |
|---|---|---|---|---|---|
| Pro-Asn | 2 | Substitution | No increase in biological activity | hEPO | US 4703008 Kiren-Amgen, Inc. |
| | 2-6 | Deletion | No increase in biological activity | hEPO | US 4703008 Kiren-Amgen, Inc. |
| Cys-His | 7 | Substitution | Eliminates biological activity | hEPO | US 4703008 Kiren-Amgen, Inc. |
| Tyr-Phe | 15 | Substitution | No increase in biological activity | hEPO | US 4703008 Kiren-Amgen, Inc. |
| | 15 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | WO 9425055 Abbott Labs. |

| | | | | | |
|---|---|---|---|---|---|
| Asn-? | 24 | Substitution | Reduces biological activity | hEPO | WO 9425055 Abbott Labs. |
| | 24 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | WO 9425055 Abbott Labs. |
| | 27-55 | Deletion | No increase in biological activity | hEPO | US 4703008 Kiren-Amgen, Inc. |
| Cys-Pro | 33 | Substitution | Loss of in-vitro activity. The disulfide bond between Cys29-Cys33 is essential for function | hEPO | WO 9425055 Abbott Labs. |
| Asn-? | 38 | Substitution | Intracellular degradation and lack of secretion | hEPO | WO 9425055 Abbott Labs. |
| Tyr-Phe | 49 | Substitution | No increase in biological activity | hEPO | US 4703008 Kiren-Amgen, Inc. |
| | 49 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | WO 9425055 Abbott Labs. |
| Met-? | 54 | Substitution | Retains in-vivo activity and is less susceptible to oxidation | hEPO | US 4835260 Genetics Institute, Inc |
| Met-Leu | 54 | Substitution | Retains biological activity | hEPO | US 4835260 Genetics Institute, Inc |
| Leu-Asn | 69 | Substitution | Creates an additional N-glycosylation site | | EP 0428267B1 AMGEN |
| | 76 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |
| | 78 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |
| | 83 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |

| | | | | | |
|---|---|---|---|---|---|
| Domain1<br><br><br>Domain2 | 99 - 119<br><br><br>111 - 129 | Deletion<br><br><br>Deletion | Rapidly degraded and inactive in-vitro<br><br><br>Retain in-vitro activity | | WO 9425055 Abbott Labs. |
| Ala-Pro | 124 | Double Substitution | Creates additional N- and O- glycosylation sites | | EP 0428267B1 AMGEN |
| Ala-Thr | 125 | Substitution | Creates additional O-glycosylation site | | EP 0428267B1 AMGEN |
| Ala-Asn<br><br>Ala-Ser | 125<br><br>127 | Double Substitution | Creates an additional N-glycosylation site<br>Creates an additional O-glycosylation site | | EP 0428267B1 AMGEN |
| Ser-? | 126 | Substitution | Rapid degradation or lack of secretion | | US 4703008 Kiren-Amgen, Inc. |
| Cys-Pro<br><br>Then<br><br>Arg-Cys | 33<br><br><br><br>139 | Double Substitution | Loss of activity<br><br><br><br>Restores and improves in-vivo activity | | WO 9425055 Abbott Labs. |
| | 143 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |
| Tyr-Phe | 145 | Substitution | No increase in biological activity | | US 4703008 Kiren-Amgen, Inc. |
| | 145 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |
| | 160 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |
| | 161 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |

| | | | | | |
|---|---|---|---|---|---|
| | 162 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |
| | 163 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |
| | 164 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |
| | 165 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |
| | 166 | Substitution or Deletion | Retains in-vivo activity in animals but there is no increase in EPO precursors | | US 4703008 Kiren-Amgen, Inc. |
| | 163 - 166 | Deletion | No increase in biological activity | | US 4703008 Kiren-Amgen, Inc. |
| Ser-? | 183 | Substitution | Intracellular degradation and lack of secretion | | US 4703008 Kiren-Amgen, Inc. |

Although hSA is the preferred fusion partner other polypeptides can be used. Preferably these are polypeptides which do not support glycosylation. The phrase "do not support glycosylation" as used herein refers to polypeptides which naturally do not support glycosylation and polypeptides which have been modified such that it does not support glycosylation. For example, the fusion partner can be a soluble fragment of Ig, preferably a soluble fragment of Ig modified such that it does not support glycosylation.

In any embodiment described herein, the hSA moiety of a fusion can be replaced with another protein, preferably a protein, e.g., a plasma protein or fragment thereof, which can improve the circulating half life of EPO or an EPOa. For example, the fusion protein can be an EPOa-immunoglobulin (Ig) fusion protein in which the EPOa sequence is fused to a sequence derived from the immunoglobulin superfamily. Several soluble fusion protein constructs have been disclosed wherein the extracellular domain of a cell surface glycoprotein is fused with the constant F(c) region of an immunoglobulin. For example, Capon et al. (1989) *Nature* 337(9):525-531, provide guidance on generating a longer lasting CD4 analog by fusing CD4 to an immunoglobulin (IgG1). *See also*, Capon et al., U.S. Patent Numbers: 5,116,964 and 5,428,130 (CD4-IgG fusion constructs); Linsley et al., U.S, Patent Number 5,434,131

(CTLA4-IgG1 and B7-IgG1 fusion constructs); Linsley et al. (1991) *J. Exp. Med.* 174:561-569 (CTLA4-IgG1 fusion constructs); and Linsley et al. (1991) *J. Exp. Med* 173:721-730 (CD28-IgG1 and B7-IgG1 fusion constructs). Such fusion proteins have proven useful for modulating receptor-ligand interactions and reducing inflammation *in vivo*. For example, fusion proteins in which an extracellular domain of cell surface tumor necrosis factor receptor (TNFR) proteins has been fused to an immunoglobulin constant (Fc) region have been used *in vivo. See, for example,* Moreland *et al.* (1997) *N. Engl. J. Med.* 337(3):141-147; and, van der Poll *et al.* (1997) *Blood* 89(10):3727-3734).

Pharmaceutical Compositions

An EPOa-hSA fusion protein or nucleic acid can be incorporated into a pharmaceutical composition useful to treat, e.g., inhibit, attenuate, prevent, or ameliorate, a condition characterized by an insufficient level of EPO activity, including conditions where the level of EPO activity is normal (but still insufficient) and those in which it is less from normal.

Preferably, the preparation of invention will be administered to a subject suffering from renal failure, chronic disease, HIV infection, blood loss or cancer, or a pre-operative patient. The compositions should contain a therapeutic or prophylactic amount of the recombinantly produced EPOa-hSA fusion protein, in a pharmaceutically-acceptable carrier or in the milk of the transgenic animal.

The pharmaceutical carrier can be any compatible, non-toxic substance suitable to deliver the polypeptides to the patient. Sterile water, alcohol, fats, waxes, and inert solids may be used as the carrier. Pharmaceutically-acceptable adjuvants, buffering agents, dispersing agents, and the like, may also be incorporated into the pharmaceutical compositions. The carrier can be combined with the EPO-hSA fusion protein in any form suitable for administration by injection (usually intravenously or subcutaneously) or otherwise. For intravenous administration, suitable carriers include, for example, physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). The concentration of the transgenically produced peptide or other active agent in the pharmaceutical composition can vary widely, i.e., from less than about 0.1% by weight, usually being at least about 1% weight to as much as 20% by weight or more.

For intravenous administration of the EPO-hSA fusion protein, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal

agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

For oral administration, the active ingredient can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. Active component(s) can be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. Examples of additional inactive ingredients that may be added to provide desirable color, taste, stability, buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide, edible white ink and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient acceptance.

For nasal administration, the polypeptides can be formulated as aerosols. The term "aerosol" includes any gas-borne suspended phase of the compounds of the instant invention which is capable of being inhaled into the bronchioles or nasal passages. Specifically, aerosol includes a gas-borne suspension of droplets of the compounds of the instant invention, as may be produced in a metered dose inhaler or nebulizer, or in a mist sprayer. Aerosol also includes a dry powder composition of a compound of the instant invention suspended in air or other carrier gas, which may be delivered by insufflation from an inhaler device, for example. See Ganderton & Jones, *Drug Delivery to the Respiratory Tract*, Ellis Horwood (1987); Gonda (1990) *Critical Reviews in Therapeutic Drug Carrier Systems* 6:273-313; and Raeburn et al. (1992) *J. Pharmacol. Toxicol. Methods* 27:143-159.

Dosage of the EPO-hSA fusion proteins of the invention may vary somewhat from individual to individual, depending on the particular peptide and its specific *in vivo* activity, the route of administration, the medical condition, age, weight or sex of the patient, the patient's sensitivities to the EPO-hSA fusion protein or components of

vehicle, and other factors which the attending physician will be capable of readily taking into account.

EPOa-hSA can be provided in a sterile container which includes dialysis solution or in a sterile container, e.g., a bag, with saline, blood, plasma, a blood substitute, or other component to be delivered to a patient.

Nutraceuticals

An EPOa-hSA fusion protein can be included in a nutraceutical. Preferably, it includes milk or milk product obtained from a transgenic mammal which expresses fusion protein. It can include plant or plant product obtained from a transgenic plant which expresses the fusion protein. The fusion protein can be provided in powder or tablet form, with or without other known additives, carriers, fillers and diluents. Nutraceuticals are described in Scott Hegenhart, Food Product Design, Dec. 1993. The nutraceutical can be an infant feeding formula. It can include components of a transgenic plant which produces an EPOa-hSA fusion protein.

Gene Therapy

EPOa-hSA constructs can be used as a part of a gene therapy protocol to deliver nucleic acids encoding an EPOa-hSA fusion protein.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, encoding a EPO-hSA fusion protein. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors can be used as a recombinant gene delivery system for the transfer of exogenous nucleic acid molecules encoding EPO-hSA fusion protein *in vivo*. These vectors provide efficient delivery of nucleic acids into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A replication defective retrovirus can be packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular

Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals.

Another viral gene delivery system useful in the present invention uses adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See, for example, Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including epithelial cells (Rosenfeld et al. (1992) cited *supra*). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267).

Another viral vector system useful for delivery of the subject nucleotide sequence encoding EPO-hSA fusion protein is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081;

Wondisford et al. (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790).

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a EPO-hSA fusion protein in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject nucleotide molecule by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a nucleic acid molecule encoding EPO-hSA fusion protein can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al. (1992) *No Shinkei Geka* 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075).

Gene delivery systems for the a gene encoding a EPO-hSA fusion protein can be introduced into a patient by any of a number of methods. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by Stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Where the fusion protein can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the fusion protein.

## Other Embodiments

### Other Transgenic Animals

EPOa-hSA fusion protein can be expressed from a variety of transgenic animals. A protocol for the production of a transgenic pig can be found in White and Yannoutsos, *Current Topics in Complement Research: 64th Forum in Immunology*, pp. 88-94; US Patent No. 5,523,226; US Patent No. 5,573,933; PCT Application WO93/25071; and PCT Application WO95/04744. A protocol for the production of a transgenic mouse can be found in US Patent No. 5,530,177. A protocol for the production of a transgenic rat can be found in Bader and Ganten, *Clinical and Experimental Pharmacology and Physiology*, Supp. 3:S81-S87, 1996. A protocol for the production of a transgenic cow can be found in *Transgenic Animal Technology, A Handbook*, 1994, ed., Carl A. Pinkert, Academic Press, Inc. A protocol for the production of a transgenic sheep can be found in *Transgenic Animal Technology, A Handbook*, 1994, ed., Carl A. Pinkert, Academic Press, Inc. A protocol for the production of a transgenic rabbit can be found in Hammer et al., *Nature* 315:680-683, 1985 and Taylor and Fan, *Frontiers in Bioscience* 2:d298-308, 1997.

Embodiments of the invention are further illustrated by the following examples which should not be construed as being limiting. The contents of all cited references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

## *Examples*

### Example 1: EPOa-hSA Fusion Constructs

The cDNA encoding the human erythropoietin analog used in the EPOa-hSA fusions was designed and engineered to alter the three N-linked and one O-linked sites of glycosylation (residues 24, 38, 83, and 126, respectively). Furthermore, without altering the remaining amino acid residues, codon usage was changed using a mammary gland protein codon usage table to maximize protein expression in the milk of transgenic animals. A schematic representation of the fusion constructs is outlined in Figure 1. In the case where hSA is the N-terminal half of the fusion protein, the hSA signal peptide was left intact and the human erythropoietin analog signal was deleted. When the human erythropoietin analog is the N-terminal part of the fusion, its signal sequence was left intact and that of the hSA protein was deleted. Also, in the first case, the wildtype hSA stop codon has been removed as was that of the human erythropoietin analog cDNA in the second construct. In addition, a linker protein $(Ser\text{-}Gly_4)_4$, or hinge, was

placed between the two fusion partners to minimize any inhibitory constraint that hSA might have on the EPO portion of the molecule and its subsequent activity.

The cDNA fusion constructs were put into the appropriate vectors for expression in tissue culture and in the mammary gland of transgenic mice. By expressing these constructs transiently in tissue culture (COS7 cells), a number of important features of the products of these cDNA fusions can be examined, e.g., (1) are the proteins being made and secreted? (2) Are these proteins authentic, recognizable by antisera against EPOa and hSA? (3) Are these proteins bioactive *in vitro* and *in vivo*?

Example 2: COS7 Cell Transfections/Western Blot Analysis

COS7 cells were transiently transfected with fusion cDNA constructs in triplicate plates or a single plate with the vector (pcDNA3) alone. Twenty-four hours after transfection, the media were replaced with a reduced serum medium (Optimem). After five days, all media were harvested and contaminating cells were removed by centrifugation. Samples of the conditioned media were then analyzed by SDS-PAGE and immunoblotting (see Figure 2).

Supernatants from COS cells transfected with HIP/pcDNA3 constructs or pcDA3 alone (mock) were analyzed by immunoblotting with a polyclonal antibody against human serum albumin (α-hSA). After analysis with the hSA antibody, the blot was stripped and reanalyzed with a monoclonal antibody against human erythropoietin (α-hEpo). The gel was loaded as follows: lane 1, 10 ng hSA standard; lane 2, 10 µl mock CM; lanes 3-5, 10 µl hSA-hEpo CM; lanes 6-8, 10 µl hEpo-hSA CM.

The results of the Western blotting experiments clearly indicate that a soluble, secreted protein was produced. Both fusion proteins are the appropriate predicted size (~89kDa). The band seen in the conditioned media lanes in the hSA antibody blot represents not hSA (~66kDa) but bSA, as this antibody has some cross reactivity with the bSA found in the tissue culture medium used. Most importantly, however, is the ability of the two antibodies to recognize both fusion proteins. This suggests that proper translation of the entire fusion construct mRNAs has been accomplished, leaving the appropriate epitopes intact and accessible to the antibodies.

Example 3: Bioactivity

An ELISA was performed with the same α-hSA antibody used in the above Western blot analysis to determine the concentrations of the two fusion proteins in the tissue culture supernatant. Consistent with the Western blot results, the EPOa-linker-hSA fusion protein was shown to be made at approximately 4-fold higher levels than the hSA-linker-EPOa fusion protein (232ng/ml versus 59ng/ml, respectively). These levels

should provide sufficient product to assess *in vitro* and, possibly, *in vivo* bioactivity. If the EPOa fraction of the fusion proteins is 20% of the total size of the molecule, 232ng/ml represents approximately 10U/ml hEpo-hSA fusion protein [$(2.1x10^5U/mg)2.32x10^{-4}mg/ml)(0.2)=9.7U/ml$]. I*n vitro* EPOa activity will be assessed using Epo-responsive cell lines. Briefly, cells are incubated 22-72 hours with increasing amounts of recombinant EPOa-hSA fusion protein and cellular growth is determined by [$^3$H]thymidine uptake or by the colorimetric MTT assay (Sigma).

EPOa-hSA fusion protein can be rapidly purified to near homogeneity using cation exchange chromatography which takes advantage of well characterized hSA binding properties. Fusion proteins can be concentrated if necessary and tested in mice. Mice can be subcutaneously injected with fusion protein (possibly with as little as 3 x 50ng/mouse, total EPOa) and responsiveness detected by determining changes in reticulocyte numbers or Hematocrit levels. Direct intramuscular injection, at high concentration (>100μg), of the pcDNA3-based plasmid DNA and subsequent monitoring of changes in reticulocyte and Hematocrit levels can be used as an *in vivo* assay. Plasmid injection has been demonstrated to significantly raise Hematocrit levels in mice when using the wildtype hEpo cDNA expressed from the cytomegalovirus promoter (CMV).

Example 4: Generation of a Erythropoietin analog-human serum albumin (EPOa-hSA) fusion protein construct

cDNA encoding EPOa-hSA fusion protein was introduced in the BC355 vector containing the regulatory elements of the goat beta-casein gene, creating a transgene having the EPOa-hSA fusion protein sequence under the control of a milk specific promoter. This construct was used to target EPOa-hSA fusion protein expression to the lactating mammary gland of a transgenic mammal.

Example 5: Testing and Characterization of Gene Constructs in Transgenic Mice

Transgene constructs are generally tested in a mouse model system to assess their ability to direct high levels of expression and their ability to express in a tissue-specific manner. Transgenic mice were generated with the expression of EPOa-hSA fusions targeted to the mammary gland.

Transgenic mice were generated by microinjecting mouse embryos with fusion protein encoding DNA constructs. Western analysis of the milk of the EPOa-hSA fusion protein transgenic mice was performed using monoclonal anti-EPO or anti-hSA antibodies to determine which animals express EPOa-hSA fusion protein in the milk.

The level of EPOa-hSA fusion protein detected ranged from about 0.2 mg/ml to 4 mg/ml.

Example 6: Bioactivity of EPOa-hSA in transgenic Mice

The bioactivity of the EPOa-hSA fusion protein was analyzed by determining changes in hematocrit levels of transgenic mice expressing EPOa-hSA fusion protein. *See* Table 1. Hematocrit levels of the transgenic mice (655-1-8, 655-1-16, 655-1-43) were compared to levels in control mice (the CD1mice). Normal hematocrit levels are about 50.

**TABLE 1**

**TRANSGENIC MICE EXPRESSING EPOA-HSA FUSION PROTEIN**

| Mouse | d.p.partum | Hematocrit | Status (10/98) |
|---|---|---|---|
| 655-1-8 | 17 | 90 | Died 7/98 |
| 655-1-16 | 16 | 86 | Died 8/98 |
| 655-1-43 | 17 | 93 | Alive |
| CD1 | 17 | 50 | NA |
| CD1 | 17 | 57 | NA |
| CD1 | 17 | 52 | NA |

As shown in Table I, expression of the EPOa-hSA fusion protein in transgenic mice significantly increased hematocrit levels in the mice.

In addition, Table II provides the hematocrit levels of virgin offspring of the founder transgenic mice and hematocrit levels for founder males (678-1-11 and 678-1-23) to demonstrate the expression of EPOa-hSA and the bioactivity of EPOa-hSA in these mice.

**TABLE II**

**HEMATOCRIT LEVELS IN VIRGIN OFFSPRING OF TRANSGENIC FOUNDER MICE EXPRESSING EPOa-hSA FUSION PROTEIN**

| Mouse | Founder | Hematocrit | Status (10/98) |
|---|---|---|---|
| 655-2-160 | 56 (low) | 50 | Alive |
| 655-2-165 | 57 (high) | 91 | Alive |
| 655-2-147 | 23 (male) | 86 | Alive |
| 678-2-155 | 31 (n.d./low) | 43 | Alive |
| 678-1-11 | (male) | | |
| 678-1-23 | (male) | | |

| | |
|---|---|
| 83 | Alive |
| 79 | Alive |

The hematocrit levels of the offspring provide basal levels of expression of EPOa-hSA under the control of a casein promoter. As shown in Table II, even low expression levels of EPOa-hSA fusion protein have a significant *in vivo* effect.

Example 7: Generation and Characterization of Transgenic Goats

The sections outlined below briefly describe the major steps in the production of transgenic goats.

Goat Species and breeds:

Swiss-origin goats, e.g., the Alpine, Saanen, and Toggenburg breeds, are preferred in the production of transgenic goats.

Goat superovulation:

The timing of estrus in the donors is synchronized on Day 0 by 6 mg subcutaneous norgestomet ear implants (Syncromate-B, CEVA Laboratories, Inc., Overland Park, KS). Prostaglandin is administered after the first seven to nine days to shut down the endogenous synthesis of progesterone. Starting on Day 13 after insertion of the implant, a total of 18 mg of follicle-stimulating hormone (FSH - Schering Corp., Kenilworth, NJ) is given intramuscularly over three days in twice-daily injections. The implant is removed on Day 14. Twenty-four hours following implant removal the donor animals are mated several times to fertile males over a two-day period (Selgrath, et al., Theriogenology, 1990. pp. 1195-1205).

Embryo collection:

Surgery for embryo collection occurs on the second day following breeding (or 72 hours following implant removal). Superovulated does are removed from food and water 36 hours prior to surgery. Does are administered 0.8 mg/kg Diazepam (Valium®) IV, followed immediately by 5.0 mg/kg Ketamine (Keteset), IV. Halothane (2.5%) is administered during surgery in 2 L/min oxygen via an endotracheal tube. The reproductive tract is exteriorized through a midline laparotomy incision. Corpora lutea, unruptured follicles greater than 6 mm in diameter, and ovarian cysts are counted to evaluate superovulation results and to predict the number of embryos that should be collected by oviductal flushing. A cannula is placed in the ostium of the oviduct and held in place with a single temporary ligature of 3.0 Prolene. A 20 gauge needle is

placed in the uterus approximately 0.5 cm from the uterotubal junction. Ten to twenty ml of sterile phosphate buffered saline (PBS) is flushed through the cannulated oviduct and collected in a Petri dish. This procedure is repeated on the opposite side and then the reproductive tract is replaced in the abdomen. Before closure, 10-20 ml of a sterile saline glycerol solution is poured into the abdominal cavity to prevent adhesions. The linea alba is closed with simple interrupted sutures of 2.0 Polydioxanone or Supramid and the skin closed with sterile wound clips.

Fertilized goat eggs are collected from the PBS oviductal flushings on a stereomicroscope, and are then washed in Ham's F12 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS) purchased from Sigma. In cases where the pronuclei are visible, the embryos is immediately microinjected. If pronuclei are not visible, the embryos can be placed in Ham's F12 containing 10% FBS for short term culture at 37°C in a humidified gas chamber containing 5% CO2 in air until the pronuclei become visible (Selgrath, et al., Theriogenology, 1990. pp. 1195-1205).

Microinjection procedure:

One-cell goat embryos are placed in a microdrop of medium under oil on a glass depression slide. Fertilized eggs having two visible pronuclei are immobilized on a flame-polished holding micropipet on a Zeiss upright microscope with a fixed stage using Normarski optics. A pronucleus is microinjected with the DNA construct of interest, e.g., a BC355 vector containing the human erythropoietin analog-human serum albumin (EPOa-hSA) fusion protein gene operably linked to the regulatory elements of the goat beta-casein gene, in injection buffer (Tris-EDTA) using a fine glass microneedle (Selgrath, et al., Theriogenology, 1990. pp. 1195-1205).

Embryo development:

After microinjection, the surviving embryos are placed in a culture of Ham's F12 containing 10% FBS and then incubated in a humidified gas chamber containing 5% CO2 in air at 37°C until the recipient animals are prepared for embryo transfer (Selgrath, et al., Theriogenology, 1990. p. 1195-1205).

Preparation of recipients:

Estrus synchronization in recipient animals is induced by 6 mg norgestomet ear implants (Syncromate-B). On Day 13 after insertion of the implant, the animals are given a single non-superovulatory injection (400 I.U.) of pregnant mares serum gonadotropin (PMSG) obtained from Sigma. Recipient females are mated to

vasectomized males to ensure estrus synchrony (Selgrath, et al., Theriogenology, 1990. pp. 1195-1205).

Embryo Transfer:

All embryos from one donor female are kept together and transferred to a single recipient when possible. The surgical procedure is identical to that outlined for embryo collection outlined above, except that the oviduct is not cannulated, and the embryos are transferred in a minimal volume of Ham's F12 containing 10% FBS into the oviductal lumen via the fimbria using a glass micropipet. Animals having more than six to eight ovulation points on the ovary are deemed unsuitable as recipients. Incision closure and post-operative care are the same as for donor animals (see, e.g., Selgrath, et al., Theriogenology, 1990. pp. 1195-1205).

Monitoring of pregnancy and parturition:

Pregnancy is determined by ultrasonography 45 days after the first day of standing estrus. At Day 110 a second ultrasound exam is conducted to confirm pregnancy and assess fetal stress. At Day 130 the pregnant recipient doe is vaccinated with tetanus toxoid and Clostridium C&D. Selenium and vitamin E (Bo-Se) are given IM and Ivermectin was given SC. The does are moved to a clean stall on Day 145 and allowed to acclimatize to this environment prior to inducing labor on about Day 147. Parturition is induced at Day 147 with 40 mg of PGF2a (Lutalyse®, Upjohn Company, Kalamazoo Michigan). This injection is given IM in two doses, one 20 mg dose followed by a 20 mg dose four hours later. The doe is under periodic observation during the day and evening following the first injection of Lutalyse® on Day 147. Observations are increased to every 30 minutes beginning on the morning of the second day. Parturition occurred between 30 and 40 hours after the first injection. Following delivery the doe is milked to collect the colostrum and passage of the placenta is confirmed.

Verification of the transgenic nature of $F_0$ animals:

To screen for transgenic $F_0$ animals, genomic DNA is isolated from two different cell lines to avoid missing any mosaic transgenics. A mosaic animal is defined as any goat that does not have at least one copy of the transgene in every cell. Therefore, an ear tissue sample (mesoderm) and blood sample are taken from a two day old $F_0$ animal for the isolation of genomic DNA (Lacy, et al., A Laboratory Manual, 1986, Cold Springs Harbor, NY; and Herrmann and Frischauf, Methods Enzymology, 1987. 152: pp. 180-183). The DNA samples are analyzed by the polymerase chain reaction (Gould, et al.,

Proc. Natl. Acad. Sci, 1989. 86:pp. 1934-1938) using primers specific for human EPOa-hSA fusion protein gene and by Southern blot analysis (Thomas, Proc Natl. Acad. Sci., 1980. 77:5201-5205) using a random primed EPO or hSA cDNA probe (Feinberg and Vogelstein, *Anal. Bioc.*, 1983. 132: pp. 6-13). Assay sensitivity is estimated to be the detection of one copy of the transgene in 10% of the somatic cells.

Generation and Selection of production herd

The procedures described above can be used for production of transgenic founder ($F_0$) goats, as well as other transgenic goats. The transgenic $F_0$ founder goats, for example, are bred to produce milk, if female, or to produce a transgenic female offspring if it is a male founder. This transgenic founder male, can be bred to non-transgenic females, to produce transgenic female offspring.

Transmission of transgene and pertinent characteristics

Transmission of the transgene of interest, in the goat line is analyzed in ear tissue and blood by PCR and Southern blot analysis. For example, Southern blot analysis of the founder male and the three transgenic offspring shows no rearrangement or change in the copy number between generations. The Southern blots are probed with human EPOa-hSA fusion protein cDNA probe. The blots are analyzed on a Betascope 603 and copy number determined by comparison of the transgene to the goat beta casein endogenous gene.

Evaluation of expression levels

The expression level of the transgenic protein, in the milk of transgenic animals, is determined using enzymatic assays or Western blots.

Other embodiments are within the following claims.

What is claimed is:

1. An EPOa-hSA fusion protein, wherein at least one amino acid residue of the EPOa moiety of the fusion protein is altered such that a site which serves as a site for glycosylation in EPO does not serve as a site for glycosylation in the EPOa.

2. The EPOa-hSA fusion protein of claim 1, wherein said fusion protein has the formula:

R1-L-R2; R2-L-R1; or R1-L-R2-L-R1,

wherein R1 is an erythropoietin analog amino acid sequence; L is a peptide linker and R2 is human serum albumin amino acid sequence.

3. The EPOa-hSA fusion protein of claim 2, wherein R1 and R2 are covalently linked via said peptide linker.

4. The EPOa-hSA fusion protein of claim 1, wherein an amino acid residue which serves as an attachment point for glycosylation has been deleted.

5. The EPOa-hSA fusion protein of claim 1, wherein an amino acid residue of human EPO which serves as a site for glycosylation has been replaced with an amino acid residue which does not serve as a site for glycosylation.

6. The EPOa-hSA fusion protein of claim 1, wherein said amino acid residue is selected from the group consisting of amino acid residues Asn24, Asn38, Asn83 and Ser126.

7. The EPOa-hSA fusion protein of claim 1, wherein said glycosylation site is altered at amino acid residue Ser126 and at least one additional N-linked glycosylation site selected from the group consisting of Asn24, Asn38 and Asn83 is altered.

8. The EPOa-hSA fusion protein of claim 1, wherein said glycosylation site provides for N-linked glycosylation and is altered by replacing an amino acid residue Asn with Gln.

9. The EPOa-hSA fusion protein of claim 1, wherein said glycosylation site provides for O-linked glycosylation and is altered by replacing an amino acid residue Ser with Gln.

10. The EPOa-hSA fusion protein of claim 1, wherein one or more of amino acid residues 24, 38, or 83 has been altered.

11. The EPOa-hSA fusion protein of claim 10, wherein one or more of amino acid residues 24, 38, or 83 has been replaced with Gln.

12. The EPOa-hSA fusion protein of claim 1, wherein amino acid residue 126 has been altered.

13. The EPOa-hSA fusion protein of claim 12, wherein said amino acid residue 126 has been replaced with Ala.

14. The EPOa-hSA fusion protein of claim 1, wherein each of amino acid residues 24, 38, 83 and 126 has been altered such that it does not serve as a glycosylation site.

15. The EPOa-hSA fusion protein of claim 14, wherein each of said amino acid residues 24, 28, 83 and 126 has been replaced with Gln, Gln, Gln, and Ala respectively.

16. The EPOa-hSA fusion protein of claim 3, wherein said peptide linker is 10 to 30 amino acids in length.

17. The EPOa-hSA fusion protein of claim 16, wherein each of said amino acids in said peptide linker is selected from the group consisting of Gly, Ser, Asn, Thr and Ala.

18. The EPOa-hSA fusion protein of claim 3, wherein said peptide linker includes a sequence having the formula $(Ser\text{-}Ser\text{-}Ser\text{-}Ser\text{-}Gly)_y$ wherein y is less than or equal to 8.

19. The EPOa-hSA fusion protein of claim 3, wherein said peptide linker includes a sequence having the formula $((Ser\text{-}Ser\text{-}Ser\text{-}Ser\text{-}Gly)_3\text{-}Ser\text{-}Pro$.

20. The EPOa-hSA fusion protein of claim 1, wherein the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

21. The EPOa-hSA fusion protein of claim 1, wherein the fusion protein includes from left to right, an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126, a peptide linker, and human serum albumin.

22. The EPOa-hSA fusion protein of claim 21, wherein the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

23. The EPOa-hSA fusion protein of claim 1, wherein the fusion protein is from left to right, Gln24, Gln38, Gln83, Ala126 EPO, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and human serum albumin.

24. The EPOa-hSA fusion protein of claim 1, wherein the EPOa-hSA fusion protein includes, from left to right, human serum albumin, a peptide linker, and an EPOa which includes amino acid residues Gln24, Gln38, Gln83 and Ala126.

25. The EPOa-hSA fusion protein of claim 24, wherein the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

26. The EPOa-hSA fusion protein of claim 1, wherein the fusion protein is from left to right, human serum albumin, a peptide linker having the formula ((Ser-Gly-Gly-Gly-Gly)$_3$-Ser-Pro), and Gln24, Gln38, Gln83, Ala126 EPO.

27. An isolated nucleic acid comprising a nucleotide sequence which encodes an EPOa-hSA fusion protein, wherein at least one amino acid residue of the encoded EPOa-hSA which can serve as a glycosylation site in EPO is altered such that it does not serve as a glycosylation site in the EPOa.

28. An expression vector or a construct which comprises the nucleic acid of claim 27.

29. A cell which comprises the vector or construct of claim 28.

30. A method of making an EPOa-hSA fusion in a construct or a vector, comprising forming in a construct or vector a sequence in which a nucleic acid which comprises a nucleotide sequence encoding an EPOa is linked in frame to a nucleic acid which comprises a nucleotide sequence encoding human serum albumin.

31. A method for making an EPOa-hSA fusion protein comprising:

supplying a cell which comprises a nucleic acid which encodes an EPOa-hSA fusion protein and

expressing said EPOa-hSA fusion protein from said nucleic acid, thereby making said EPOa-hSA fusion protein.

32. The method of claim 31, wherein said cell is selected from a group consisting of a mammalian, yeast, plant, insect or a bacterial cell.

33. A method of making an EPOa-hSA fusion protein comprising:

providing a transgenic organism which includes a transgene which directs the expression of EPOa-hSA fusion protein;

allowing the transgene to be expressed; and

recovering EPOa-hSA fusion protein.

34. The method of claim 33 wherein, the transgenic organism is a transgenic animal.

35. The method of claim 33 wherein, the transgenic organism is a transgenic dairy animal.

36. The method of claim 33 wherein, the EPOa-hSA fusion protein is made in a mammary gland of a transgenic mammal under the control of a milk specific promoter.

37. The method of claim 36 wherein, said promoter is a milk serum protein or casein promoter.

38. The method of claim 37 wherein, the transgenic mammal is a goat.

39. A method for providing a transgenic preparation which includes an EPOa-hSA fusion protein in the milk of a transgenic mammal comprising:

providing a transgenic mammal having an EPOa-hSA fusion protein protein-coding sequence operatively linked to a promoter sequence that results in the expression of the protein-coding sequence in mammary gland epithelial cells,

allowing the fusion protein to be expressed, and obtaining milk from the mammal, thereby providing the transgenic preparation.

40. A transgenic organism, which includes a transgene which encodes an EPOa-hSA fusion protein.

41. The method of claim 40 wherein, the transgenic organism is a transgenic animal.

42. The method of claim 40 wherein, the transgenic organism is a transgenic dairy animal.

43. The method of claim 40 wherein, the EPOa-hSA fusion protein is made in a mammary gland of a transgenic mammal under the control of a milk specific promoter.

44. The method of claim 43 wherein, said promoter is a milk serum protein or casein promoter.

45. The method of claim 44 wherein, the transgenic mammal is a goat or cow.

46. A pharmaceutical composition having a therapeutically effective amount of an EPOa-hSA fusion protein.

47. A method of treating a subject in need of erythropoietin comprising administering a therapeutically effective amount of an EPOa-hSA fusion protein to the subject.

48. An erythropoietin analog, wherein four sites which serve as sites for glycosylation in erythropoietin are altered such that they do not serve as glycosylation sites.

49. The erythropoietin analog of claim 48 wherein the EPOa is Gln24, Gln38, Gln83, Ala126 EPO.

50. A transgenic rabbit, which includes a transgene which encodes an EPOa-hSA fusion protein.

51. A bird, which includes a transgene which encodes an EPOa-hSA fusion protein.

52. A method for making an EPOa-hSA fusion protein in a cultured cell comprising supplying a cell which includes a nucleic acid which encodes an EPOa-hSA fusion protein, and expressing the EPOa-hSA fusion protein from the nucleic acid, thereby making the EPOa-hSA fusion protein.

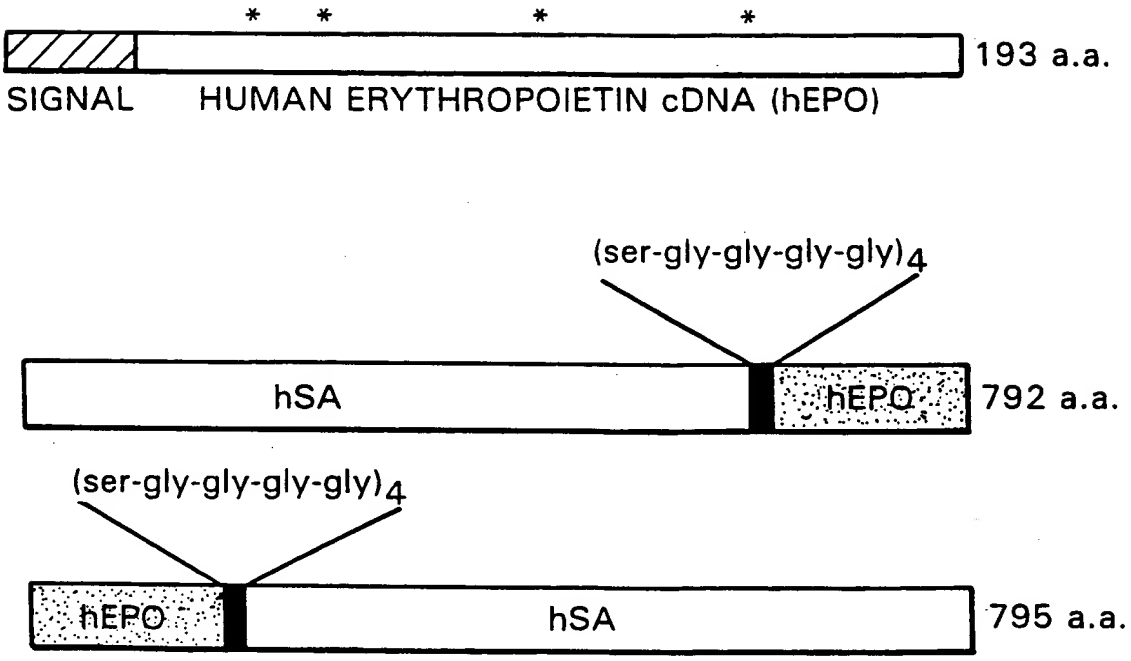
193 a.a.
SIGNAL
HUMAN ERYTHROPOIETIN cDNA (hEPO)
(ser-gly-gly-gly-gly)4
hSA
hEPO
792 a.a.
(ser-gly-gly-gly-gly)4
hEPO
hSA
795 a.a.

FIG. 1

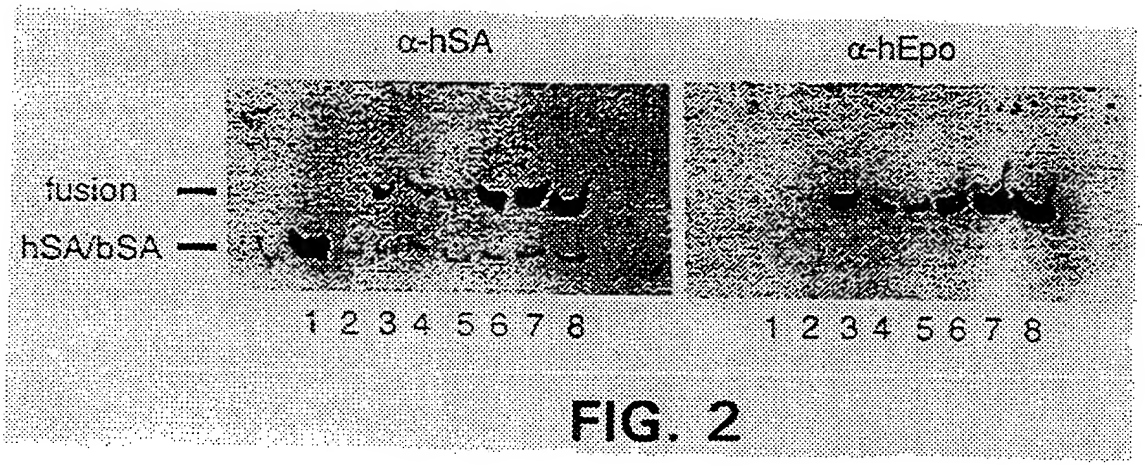


FIG. 2